

LAW OFFICES
SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC
2100 PENNSYLVANIA AVENUE, N.W.
WASHINGTON, DC 20037-3213
TELEPHONE (202) 293-7060
FACSIMILE (202) 293-7860
www.sughrue.com

October 16, 2000

BOX PCT

Assistant Commissioner
for Patents
Washington, D.C. 20231

PCT/AU99/00306 - filed
April 23, 1999

Re: Application of **Susan J. CLARK,**
Douglas S. MILLAR and **Peter L. MOLLOY**
entitled "ASSAY FOR METHYLATION IN
THE GST-Pi GENE"
Our Ref: Q-61152

Dear Sir:

The following documents and fees are submitted herewith in connection with the above application for the purpose of entering the National stage under 35 U.S.C. § 371 and in accordance with Chapter II of the Patent Cooperation Treaty:

- ☐ an executed Declaration and Power of Attorney.
- ☒ the International Application (in English).
- ☒ 17 sheets of drawings.
- ☒ Substitute pages 5-11 and 62-72.
- ☐ an English translation of Article 19 claim amendments.
- ☒ an International Preliminary Examination Report.
- ☒ Statement in Support of Submission (along with substitute Sequence Listing and DOS version diskette containing the same).
- ☐ an executed Assignment and PTO 1595 form.
- ☒ a Form PTO-1449 listing the ISR references and a complete copy of each reference.
- ☐ Written Opinion.
- ☒ a Preliminary Amendment.

SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC

Assistant Commissioner
of PatentOctober 16, 2000
Page 2

The Declaration and Power of Attorney and Assignment will be submitted at a later date.

Priority is claimed from April 23, 1998, based on Australian Application No. PP 3129.

The Government filing fee is calculated as follows:

Total claims	<u>50</u> - <u>20</u> = <u>30</u>	x	\$18.00	=	<u>\$540.00</u>
Independent claims	<u>4</u> - <u>3</u> = <u>1</u>	x	\$80.00	=	<u>\$80.00</u>
Base Fee					<u>\$1000.00</u>
Multiple Dependent Claim Fee					<u>\$0.00</u>

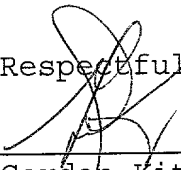
TOTAL FEE\$1620.00

A check for the statutory filing fee of \$1620.00 will be submitted shortly.

The Assistant Commissioner is hereby directed and authorized to charge or credit any difference or overpayment to Deposit Account No. 19-4880.

The Assistant Commissioner is also hereby authorized to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.492 which may be required during the entire pendency of the application to Deposit Account No. 19-4880. A duplicate copy of this transmittal letter is attached.

Respectfully submitted,



 Gordon Kit

Registration No. 30,764

**SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC**

2100 Pennsylvania Avenue, N.W.
Washington, D.C. 20037-3213
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

Date: October 16, 2000

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Susan J. CLARK et al

Appln. No.: 09/673,448

Filed: October 16, 2000

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE



Group Art Unit: 0000

Examiner: Unknown

STATEMENT IN SUPPORT OF SUBMISSION
IN ACCORDANCE WITH 37 C.F.R. § 1.821

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

The following statement is provided to meet the requirements of 37 C.F.R. § 1.821.

I hereby state that the content of the computer readable copy (PatentIn Version 3.0) of the third substitute Sequence Listing (attached hereto) submitted in accordance with 37 C.F.R. §§ 1.821(c) and (e), respectively, is the same as the third substitute Sequence Listing submitted herewith.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

7/12/01

Date


Gordon Kit

10 MAY 2001

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Susan J. CLARK et al

Appln. No.: 09/673,448

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-PI GENE

STATEMENT IN SUPPORT OF SUBMISSION
IN ACCORDANCE WITH 37 C.F.R. § 1.821

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

The following statement is provided to meet the requirements of 37 C.F.R. § 1.821.

I hereby state that the content of the computer readable copy (PatentIn Version 3.0) of the substitute Sequence Listing submitted in accordance with 37 C.F.R. §§ 1.821(c) and (e), respectively, is the same as the substitute Sequence Listing filed simultaneously herewith.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that

[illegible]

5/10/01

Date

Gordon Kit

09/673448

532 Rec'd PCT/PTC 16 OCT 2000

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

SUSAN J. CLARK et al

CHAPT II filing
Appln. No.: of PCT/AU99/00306

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE

STATEMENT IN SUPPORT OF SUBMISSION
IN ACCORDANCE WITH 37 C.F.R. § 1.821

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

The following statement is provided to meet the requirements of 37 C.F.R. § 1.821.

I hereby state that the content of the computer readable copy (PatentIn Version 2.1) of the substitute Sequence Listing submitted in accordance with 37 C.F.R. §§ 1.821(c) and (e), respectively, is the same as the substitute Sequence Listing filed simultaneously herewith.


I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that

STATEMENT IN SUPPORT OF SUBMISSION
CHAPTER II FILING OF PCT/AU99/00306

such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

10/6/00

Date



Gordon Kit

09/673448

532 Rec'd PCT/PTC 16 OCT 2000

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

SUSAN J. CLARK et al

CHAPT II filing

Appln. No.: of PCT/AU99/00306

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE

PRELIMINARY AMENDMENT

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

Prior to examining the above-identified application, please
amend the application as follows.

IN THE SPECIFICATION:

Page 1, before line 3, insert

-- This application is a 371 of PCT/AU99/00306 filed
April 23, 1999. --

Pages 5-11, delete in their entirety, and insert therefor
substitute pages 5-11 filed simultaneously herewith.

Substitute pages 62-72 (filed simultaneously herewith),
please renumber as pages 45-55, respectively.

IN THE CLAIMS:

Please amend the claims (substitute Claims 1-50, i.e.,
substitute pages 62-72, filed simultaneously herewith) as
follows:

PRELIMINARY AMENDMENT
CHAPTER II Filing
of PCT/AU99/00306

Claim 3, line 1, change "any one of the preceding claims"
to -- Claim 1 --.

Claim 14, line 1, change "any one of the preceding claims"
to -- Claim 1 --.

Claim 15, line 1, change "any one of the preceding claims"
to -- Claim 1 --.

Claim 22, line 1, change "any one of the preceding claims"
to -- Claim 1 --.

Claim 23, line 1, change "any one of claims 5 to 21" to
-- Claim 5 --.

Claim 37, line 1, delete "or 36".

Claim 40, line 1, change "any one of claims 35 to 39" to
-- Claim 35 --.

Claim 42, line 1, change "any one of claims 35 to 41" to
-- Claim 35 --.

Claim 44, line 1, change "any one of claims 35 to 43" to
-- Claim 35 --.

Claim 45, line 1, change "any one of claims 35 to 43" to
-- Claim 35 --.

IN THE SEQUENCE LISTING:

Pages 45-61 (Sequence Listing), please in its entirety.

IN THE ABSTRACT:

Please insert the Abstract attached hereto.

PRELIMINARY AMENDMENT
CHAPTER II Filing
of PCT/AU99/00306

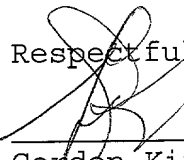
REMARKS

The specification has been amended to insert formal matter, the claims have amended to delete their multiply dependency and the Abstract has been inserted in order to make the application consistent with U.S. patent practice. Hence, the amendment of the specification and claims and the addition of the Abstract does not constitute new matter.

The Examiner is requested to note that Applicants simultaneously file herewith a substitute Sequence Listing (which is now considered to be a separate document by the U.S. Patent and Trademark Office) in PatentIn Version 2.1. Hence, the present Sequence Listing in the above-identified application is not necessary.

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,



Gordon Kit
Registration No. 30,764

SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC
2100 Pennsylvania Avenue, N.W.
Washington, D.C. 20037-3202
(202) 293-7060

Date: October 16, 2000

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Susan J. CLARK et al

Appln. No.: 09/673,448

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE

SECOND PRELIMINARY AMENDMENT

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

Further to the Preliminary Amendment of October 16, 2000,
and prior to examining the above-identified application, please
amend the application as follows.

IN THE SEQUENCE LISTING:

Please delete the present substitute Sequence Listing and
insert therefor the attached second substitute Sequence Listing.


REMARKS

Applicants simultaneously file herewith a second substitute
Sequence Listing (in order to correct the formatting errors as
noted in the Notification of Missing Requirements dated
April 19, 2001) in PatentIn Version 3.0. Hence, the submission of
the present second substitute Sequence Listing does not raise new
issues or constitute new matter.

SECOND PRELIMINARY AMENDMENT
U.S. Appln. No. 09/673,448

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,



Gordon Kilt

Registration No. 30,764

SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC
2100 Pennsylvania Avenue, N.W.
Washington, D.C. 20037-3202
(202) 293-7060

Date: May 10, 2001



PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Susan J. CLARK et al

Appln. No.: 09/673,448

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE

THIRD PRELIMINARY AMENDMENT

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

Further to the Second Preliminary Amendment filed May 10, 2001, and the Preliminary Amendment of October 16, 2000, and prior to examining the above-identified application, please amend the application as follows.

IN THE SEQUENCE LISTING:

Please delete the present second substitute Sequence Listing and insert therefor the attached third substitute Sequence Listing.

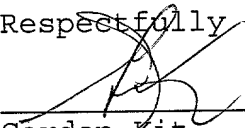
REMARKS

Applicants simultaneously file herewith a third substitute Sequence Listing (in order to correct the errors noted in the Raw Sequence Listing attached to the Notification to Comply with Requirements dated June 29, 2001) in PatentIn Version 3.0. Hence, the submission of the present third substitute Sequence Listing does not raise new issues or constitute new matter.

THIRD PRELIMINARY AMENDMENT
U.S. Appln. No. 09/673,448

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,



Gordon Kit

Registration No. 30,764

SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC
2100 Pennsylvania Avenue, N.W.
Washington, D.C. 20037-3202
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

Date: July 12, 2001

Sequence Listings:

Applicant: Commonwealth Scientific and Industrial Research
Organisation

Title: Diagnostic assay

Prior Application Number: PP3129

Prior Application Filing Date: 1998-04-23

Number of SEQ ID NOs: 59

Software: PatentIn Ver. 2.1

SEQ ID NO: 1

Length: 29

Type: DNA

Organism: Homo sapiens

Sequence: 1

cgcgagggttt tcgttgaggt ttcgtcgtc 29

SEQ ID NO: 2

Length: 25

Type: DNA

Organism: Homo sapiens

Sequence: 2

cgttattagt gactacgcgc gggttc 25

SEQ ID NO: 3

Length: 24

Type: DNA

Organism: Homo sapiens

2/17

Sequence: 3

yggtttttagg gaattttttt tcgc

24

SEQ ID NO: 4

Length: 28

Type: DNA

Organism: Homo sapiens

Sequence: 4

ygggygygtta gtttggttgyg tatatttc

28

SEQ ID NO: 5

Length: 29

Type: DNA

Organism: Homo sapiens

Sequence: 5

gggaattttt tttcgcgatg ttttyggcgc

29

SEQ ID NO: 6

Length: 24

Type: DNA

Organism: Homo sapiens

Sequence: 6

tttttagggg gtttyggagcg tttc

24

SEQ ID NO: 7

Length: 19

Type: DNA

Organism: Homo sapiens

Sequence: 7

ggtaggttgy gtttatcgc

19

SEQ ID NO: 8

3/17

Length: 27

Type: DNA

Organism: Homo sapiens

Sequence: 8

aaaaattcra atctctccga ataaacg

27

SEQ ID NO: 9

Length: 27

Type: DNA

Organism: Homo sapiens

Sequence: 9

aaaaaccraa ataaaaacca cacgacg

27

SEQ ID NO: 10

Length: 25

Type: DNA

Organism: Homo sapiens

Sequence: 10

tcccatccct ccccgaaacg ctccg

25

SEQ ID NO: 11

Length: 33

Type: DNA

Organism: Homo sapiens

Sequence: 11

gaaacgctcc gaacccccta aaaaccgcta acg

33

SEQ ID NO: 12

Length: 27

Type: DNA

Organism: Homo sapiens

4/17

Sequence: 12

crccctaataa tccccraaat crccgcg

27

SEQ ID NO: 13

Length: 30

Type: DNA

Organism: Homo sapiens

Sequence: 13

accccracra ccrctacacc ccraacgtcg

30

SEQ ID NO: 14

Length: 31

Type: DNA

Organism: Homo sapiens

Sequence: 14

ctcttctaaa aaatcccr cr aactcccgcc g

31

SEQ ID NO: 15

Length: 29

Type: DNA

Organism: Homo sapiens

Sequence: 15

aaaacrcctt aaaatccccg aaatcgccg

29

SEQ ID NO: 16

Length: 30

Type: DNA

Organism: Homo sapiens

Sequence: 16

aactcccrcc gacccaacc ccgacgaccg

30

SEQ ID NO: 17

5/17

Length: 23

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds bisulfite-converted human GST-Pi gene

Sequence: 17

aaacctaataa aataaacaaa caa

23

SEQ ID NO: 18

Length: 23

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds non-converted human GST-Pi gene

Sequence: 18

gggcctaggg agtaaacaga cag

23

SEQ ID NO: 19

Length: 25

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds human GST-Pi gene

Sequence: 19

6/17

cctttccctc ttcccarrrt cccca

25

SEQ ID NO: 20

Length: 25

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds bisulfite-converted human GST-Pi gene

Sequence: 20

tttggtattt tttttcgggt tttag

25

SEQ ID NO: 21

Length: 25

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds non-converted human GST-Pi gene

Sequence: 21

cttgcatcc tcccccgggc tccag

25

SEQ ID NO: 22

Length: 26

Type: DNA

Organism: Artificial Sequence

Feature:

7/17

Other Information: Description of Artificial
Sequence: Oligonucleotide
which binds human GST-Pi gene

Sequence: 22

gggaggggaag ggaggyaggg gytggg

26

SEQ ID NO: 23

Length: 31

Type: DNA

Organism: Homo sapiens

Sequence: 23

ttatgtaata aatttgata tttgtatat g

31

SEQ ID NO: 24

Length: 25

Type: DNA

Organism: Homo sapiens

Sequence: 24

tgtagattat ttaaggtag gagtt

25

SEQ ID NO: 25

Length: 27

Type: DNA

Organism: Homo sapiens

Sequence: 25

aaacctaataa aataaataa caacaaa

27

SEQ ID NO: 26

Length: 29

Type: DNA

Organism: Homo sapiens

8/17

Sequence: 26

aaaaaacctt tccctctttc ccaaattccc

29

SEQ ID NO: 27

Length: 27

Type: DNA

Organism: Homo sapiens

Sequence: 27

tttggtgttt gtttattttt taggttt

27

SEQ ID NO: 28

Length: 26

Type: DNA

Organism: Homo sapiens

Sequence: 28

gggatttggg aaagagggaa aggttt

26

SEQ ID NO: 29

Length: 24

Type: DNA

Organism: Homo sapiens

Sequence: 29

actaaaaact ctaaacccca tccc

24

SEQ ID NO: 30

Length: 24

Type: DNA

Organism: Homo sapiens

Sequence: 30

aacctaatat taccttaacc ccat

24

9/17

SEQ ID NO: 31

Length: 33

Type: DNA

Organism: Homo sapiens

Sequence: 31

aatcctcttc ctactatcta tttactccct aaa 33

SEQ ID NO: 32

Length: 29

Type: DNA

Organism: Homo sapiens

Sequence: 32

aaaacctaaa aaaaaaaaaa aaacttccc 29

SEQ ID NO: 33

Length: 29

Type: DNA

Organism: Homo sapiens

Sequence: 33

ttgggttttat gttgggagtt ttgagtttt 29

SEQ ID NO: 34

Length: 29

Type: DNA

Organism: Homo sapiens

Sequence: 34

ttttgtgggg agttgggggtt tgatgttgt 29

SEQ ID NO: 35

Length: 29

Type: DNA

Organism: Homo sapiens

10/17

Sequence: 35
ggtttagagt ttttagtatg gggttaatt 29

SEQ ID NO: 36
Length: 20
Type: DNA
Organism: Homo sapiens

Sequence: 36
tagtattagg ttagggtttt 20

SEQ ID NO: 37
Length: 29
Type: DNA
Organism: Homo sapiens

Sequence: 37
aactctaacc ctaatctacc aacaacata 29

SEQ ID NO: 38
Length: 29
Type: DNA
Organism: Homo sapiens

Sequence: 38
caaaaaactt taaataaacc ctcctacca 29

SEQ ID NO: 39
Length: 32
Type: DNA
Organism: Homo sapiens

Sequence: 39
gttttggtggt taggttggtt tttaggtggt ag 32

11/17

SEQ ID NO: 40

Length: 30

Type: DNA

Organism: Homo sapiens

Sequence: 40

gttttgagta tttgttgtgt ggtagttttt 30

SEQ ID NO: 41

Length: 30

Type: DNA

Organism: Homo sapiens

Sequence: 41

ttaatataaa taaaaaaaaat atatttacia 30

SEQ ID NO: 42

Length: 34

Type: DNA

Organism: Homo sapiens

Sequence: 42

caacccccaa tacccaaccc taatacaaat actc 34

SEQ ID NO: 43

Length: 26

Type: DNA

Organism: Homo sapiens

Sequence: 43

ggtttttagtt tttggttggt tggatg 26

SEQ ID NO: 44

Length: 26

Type: DNA

12/17

Organism: Homo sapiens

Sequence: 44

tttttttggt tttagtatat gtgggg 26

SEQ ID NO: 45

Length: 30

Type: DNA

Organism: Homo sapiens

Sequence: 45

atactaaaaa aactattttc taatcctcta 30

SEQ ID NO: 46

Length: 29

Type: DNA

Organism: Homo sapiens

Sequence: 46

cctaaactaaa aactcctaaa aaccactaa 29

SEQ ID NO: 47

Length: 38

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 47

tgtaaaacga cggccagtgg gatttgggaa agaggga 38

SEQ ID NO: 48

Length: 38

Type: DNA

13/17

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 48

tgtaaaacga cggccagttg ttgggagttt tgagtttt 38

SEQ ID NO: 49

Length: 31

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 49

tgtaaaacga cggccagtta gtattaggtt a 31

SEQ ID NO: 50

Length: 37

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 50

tgtaaaacga cggccagtgt tttgagtatt tgttgtg 37

SEQ ID NO: 51

Length: 35

Type: DNA

14/17

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 51

tgtaaaacga cggccagtgt ttttagtata tgtgg 35

SEQ ID NO: 52

Length: 499

Type: DNA

Organism: Homo sapiens

Sequence: 52

tgcagatcac ctaagggtcag gagttcgaga ccagcccggc caacatgggtg aaaccccgtc 60
tctactaaaa atacaaaaat cagccagatg tggcagcgac ctataattcc acctactcgg 120
gaggctgaag cagaattgct tgaacccgag aggcggaggt tgcagtgagc cgccgagatc 180
gcgccactgc actccagcct gggccacagc gtgagactac gtcataaaat aaaataaaat 240
aacacaaaaat aaaataaaat aaaataaaat aaaataaaat aataaaaataa aataaaaataa 300
aataaaaataa aataaaaataa agcaatttcc tttcctctaa gcggcctcca cccctctccc 360
ctgccctgtg aagcgggtgt gcaagctccg ggatcgcagc ggtcttaggg aatttccccc 420
cgcgatgtcc cggcgcgcga gttcgctgcg cacacttcgc tgcggtcctc ttctgctgt 480
ctgtttactc cctaggccc 499

SEQ ID NO: 53

Length: 316

Type: DNA

Organism: Homo sapiens

Sequence: 53

gggacctggg aaagaggggaa aggcttcccc ggccagctgc gcggcgactc cggggactcc 60
agggcgcccc tctgcggccg acgcccgggg tgcagcggcc gccggggctg gggcggcgcg 120
gagtccgcgg gaccctccag aagagcggcc ggcgcctga ctcagcactg gggcggagcg 180
gggcgggacc acccttataa ggctcggagg ccgcgaggcc ttcgctggag ttgcgcgcgc 240
gcagtcttcg ccaccagtga gtacgcgcgg ccgcgctccc cggggatggg gctcagagct 300

15/17

cccagcatgg ggccaa

316

SEQ ID NO: 54

Length: 603

Type: DNA

Organism: Homo sapiens

Sequence: 54

cagcatcagg cccgggctcc cggcagggt cctcgccac ctcgagacc gggacggggg 60
cctaggggac ccaggacgtc ccagtgccg ttagcggctt tcagggggcc cggagcgcct 120
cggggaggga tgggaccccg ggggcgggga gggggggcag gctgcgctca ccgcgccttg 180
gcatcctccc ccgggctcca gcaaactttt ctttgttcgc tgcagtgccg ccctacaccg 240
tggtctatatt ccagttcga ggtaggagca tgtgtctggc agggaaaggga ggcagggggt 300
ggggctgcag ccacagccc ctcgcccacc cggagagatc cgaaccccct tatccctccg 360
tcgtgtgggt tttaccccg gctccttcc tgttccccgc ctctcccgcc atgctgtctc 420
cccggcccag tggtgtgtga aatcttcgga ggaacctgtt tacctgttcc ctccctgcac 480
tcctgacccc tccccgggtt gctgcgaggc ggagtcggcc cgggtccccc atctcgtact 540
tctccctccc cgcaggccgc tgcgcggccc tgcgcatgct gctggcagat cagggccaga 600
gct 603

SEQ ID NO: 55

Length: 266

Type: DNA

Organism: Homo sapiens

Sequence: 55

gctctgagca cctgctgtgt ggcagtctct catccttcca cgcacatcct cttccctcc 60
tcccaggctg gggctcacag acagccccct ggttgccca tcccagtga ctgtgtgttg 120
atcaggcgcc cagtcacgag gctgctccc ctccaccaa ccccagggt ctatgggaag 180
gaccagcagg aggcagccct ggtggacatg gtgaatgacg gcgtggagga cctccgctgc 240
aaatacatct cctcatcta caccaa 266

SEQ ID NO: 56

Length: 287

Type: DNA

Organism: Homo sapiens

16/17

Sequence: 56

tccccctgct ctacagcatat gtggggcgcc tcagtgcccg gcccaagctc aaggccttcc 60
tggcctcccc tgagtacgtg aacctcccca tcaatggcaa cgggaaacag tgagggttgg 120
ggggactctg agcgggaggg agagtttgcc ttcctttctc caggaccaat aaaatttcta 180
agagagctac tatgagcaact gtgtttcctg ggacggggct taggggttct cagcctcgag 240
gtcgggtggga gggcagagca gaggactaga aaacagctcc tccagca 287

SEQ ID NO: 57

Length: 524

Type: DNA

Organism: Homo sapiens

Sequence: 57

ataaaataaa ataaaataaa ataaagcaat ttcctttcct ctaagcggcc tccacccctc 60
tccctgccc tgtgaagcgg gtgtgcaagc tccgggatcg cagcggctct aggaatttc 120
ccccgcgat gtccggcgcc gccagttcgc tgcgcacact tcgctgcggt cctcttctcg 180
ctgtctgttt actccctagg ccccgctggg gacctgggaa agagggaaag gcttcccccg 240
ccagctgcgc ggcgactccg gggactccag ggcgcccctc tgcggccgac gcccggggtg 300
cagcggccgc cggggctggg gccggcgggg gtccgcggga ccctccagaa gagcggcccg 360
cgccgtgact cagcactggg gcggagcggg gcgggaccac ccttataagg ctcggaggcc 420
gcgaggcctt cgctggagtt tcgccgcgc agtcttcgcc accagtgagt acgcgcggcc 480
cgcgccccg gggatggggc tcagagctcc cagcatgggg ccaa 524

SEQ ID NO: 58

Length: 524

Type: DNA

Organism: Homo sapiens

Sequence: 58

ataaaataaa ataaaataaa ataaagtaat tttttttttt ttaagtgggt tttatttttt 60
ttttttgttt tgtgaagtgg gtgtgtaagt tttgggattg tagtggtttt aggaatttt 120
tttttgatgt gttttggtgt gttagtttgt tgtgtatatt ttgttggtgt tttttttttg 180
ttgtttgttt attttttagg ttttgttggg gatattggaa agagggaaag gttttttttg 240
ttagttgtgt ggtgattttg gggatttttag ggtgtttttt tgtggttgat gtttgggggt 300
tagtggttgt tgggggttgg gtttgtggga gtttttagaa gagtgggttg 360

17/17

tgttgtgatt tagtattggg gtggagtggg gtgggattat ttttataagg tttggagggt 420
gtgagggttt tgttgagggt ttgttgttgt agtttttgtt attagtgagt atgtgtgggt 480
tgtgtttttg gggatggggg ttagagtttt tagtatgggg ttaa 524

SEQ ID NO: 59

Length: 524

Type: DNA

Organism: Homo sapiens

Sequence: 59

ataaaataaa ataaaataaa ataaagtaat tttttttttt ttaagcgggt tttatttttt 60
ttttttgttt tgtgaagcgg gtgtgtaagt ttcgggatcg tagcgggttt aggaatttt 120
ttttcgcgat gtttcggcgc gttagtctgt tgcgtatatt tcgttcgggt tttttttttg 180
ttgtttgttt attttttagg tttcgttggg gatttgggaa agagggaaaag gttttttcgg 240
ttagttgcgc ggcgatttcg gggatttttag ggcgtttttt tgcggtcgac gttcgggggtg 300
tagcggtcgt cggggttggg gtcggcggga gttcgcggga ttttttagaa gagcggtcgg 360
cgtcgtgatt tagtattggg gcggagcggg gcgggattat ttttataagg ttcggagggtc 420
gcgaggtttt cgttgagggt tcgtcgtcgt agttttcgtt attagtgagt acgcgcgggt 480
cgcgttttcg gggatggggg ttagagtttt tagtatgggg ttaa 524

ASSAY FOR METHYLATION IN THE GST-PI GENE**Field of the Invention:**

This invention relates to an assay for diagnosis or prognosis of a disease or condition characterised by abnormal methylation of cytosine at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its regulatory flanking sequences. In one particular application, the invention provides an assay for the diagnosis or prognosis of prostate cancer.

Background of the Invention:*DNA METHYLATION IN MAMMALIAN GENOMES*

The only established post-synthetic modification of DNA in higher animal and plant genomes is methylation of the 5' position of cytosine. The proportion of cytosines which are methylated can vary from a few percent in some animal genomes (1) to 30% in some plant genomes (2). Much of this methylation is found at CpG sites where the symmetrically positioned cytosines on each strand are methylated. In plant genomes, similar symmetrical methylation of cytosines at CpNpG (where N can be any base) is also common (3). Such sites of methylation have also been identified at low frequency in mammalian DNA (4).

Methylation patterns are heritable as the methylase enzyme recognises as a substrate, sites where a CpG dinucleotide is methylated on one strand but the corresponding C on the other strand is unmethylated, and proceeds to methylate it (5, 6). Fully unmethylated sites do not normally act as substrates for the enzyme and hence remain unmethylated through successive cell divisions. Thus, in the absence of errors or specific intervening events, the methylase enzyme enables the stable heritability of methylation patterns.

Extensive studies of gene expression in vertebrates have shown a strong correlation between methylation of regulatory regions of genes and

their lack of expression (7). Most of such studies have examined only a limited number of restriction enzyme sites using enzymes which fail to cut if their target sites are methylated. A far more limited number have been examined at all cytosine bases using genomic sequencing methods (8, 9).

5 *BISULPHITE CONVERSION OF DNA*

Treatment of single-stranded DNA with high concentrations of bisulphite followed by alkali leads to the selective deamination of cytosine, converting it to uracil (10, 11). By contrast, 5-methyl cytosines (5meC) are resistant to this chemical deamination. When bisulphite-treated DNA is copied by DNA polymerases, the uracils are read as if they were thymines and an adenine nucleotide incorporated, while 5meC is still read as a cytosine (a G being incorporated opposite). Thus, after a region of sequence is amplified by polymerase chain reaction (PCR), cytosines in the sequence which were methylated in the original DNA will be read as cytosines while unmethylated cytosines will be read as thymines (12, 13).

15 *PCR AMPLIFICATION OF METHYLATED AND UNMETHYLATED DNA*

In order to amplify bisulphite-treated DNA, primers are designed to anneal to the sequence produced after bisulphite treatment of the DNA. Since cytosines are converted to uracils, the base in the annealing primer will be an adenine rather than a guanine for the non-converted cytosine. Similarly, for the other primer of the pair, thymines replace cytosines. To permit quantification of levels of methylation in the target DNA, primers are normally chosen to avoid sites which may or may not be methylated (particularly CpG sites) and so may contain either a 5meC or a uracil after bisulphite treatment. Use of such non-selective primers allows both methylated and unmethylated DNAs to be amplified by PCR, providing for quantification of the level of methylation in the starting DNA population. The PCR-amplified DNA can be cut with an informative restriction enzyme, can be sequenced directly to provide an average measure of the proportion of methylation at any position or molecules may be cloned and sequenced (each

clone will be derived from amplification of an individual strand in the initial DNA). Such studies have indicated that, while a population of molecules may conform to an overall pattern of methylation, not all molecules will be identical and methylation may be found on only a fraction of molecules at some sites (13, 16).

SELECTIVE AMPLIFICATION OF METHYLATED DNA

Recently Herman *et al.* (14) described a variation of the bisulphite sequencing procedure to make it selective for the amplification of only methylated DNA. In this work, PCR primers were used which were designed to discriminate between the sequences produced after bisulphite-treatment of methylated and non-methylated target DNAs. Thus, cytosines which formed part of a CpG site would not be bisulphite converted and would remain as cytosines in the methylated DNA but would be converted to uracils in the unmethylated target DNA. Primers utilising these differences were designed and used for the amplification of methylated DNA sequences from four tumour suppressor genes, p16, p15, E-cadherin and von Hippel-Lindau.

METHYLATION OF THE GLUTATHIONE-S-TRANSFERASE Pi GENE IN PROSTATE CANCER

Lee *et al.* (15) (US Patent No 5,552,277 and International Patent Application No PCT/US95/09050) demonstrated that expression of the glutathione-S-transferase (GST) Pi gene is lost in nearly all cases of prostate cancer. They further showed that in twenty cases examined, using Southern blotting, that this loss of expression was accompanied by methylation at a specific restriction enzyme site (*Bss*HII) in the promoter region of the gene. This methylation was not seen in normal prostate tissue or in a number of other normal tissues examined. In examining a prostate cancer cell line in which the GST-Pi gene is inactive, they also identified methylation at two other restriction enzyme sites, *Not*I and *Sac*II in the promoter region of the gene. Digestion of cell line DNAs with the enzymes *Msp*I and *Hpa*II, indicated that the correlation of DNA methylation with lack of expression

was not maintained for these sites which were largely located downstream of the transcription start site. The nature of the data makes it difficult to reach conclusions on the methylation status of individual *MspI/HpaII* sites.

However, Lee *et al.* (18) were able to show that following *HpaII* digestion (which will cut at all unmethylated *HpaII* sites), a region of DNA containing twelve *HpaII* recognition sites could be amplified by PCR from tumour DNA, but not from normal prostate or leukocyte DNA. This indicates that some DNA molecules in prostate cancer are methylated at all these *HpaII* sites, while DNAs from normal prostate and leukocyte DNA must contain at least one of these sites unmethylated (as a single cut will render the region incapable of being amplified by PCR).

The present inventors have identified and developed an alternative method for detecting sites of methylation present in DNA from prostate cancer tissue but not present in DNA from normal tissue. The method relies on selective amplification of a target region of the GST-Pi gene but does not require prior restriction with an informative restriction enzyme.

Disclosure of the Invention:

Thus, in a first aspect, the present invention provides a diagnostic or prognostic assay for a disease or condition in a subject, said disease or condition characterised by abnormal methylation of cytosine at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its regulatory flanking sequences, wherein said assay comprises the steps of;

- (i) isolating DNA from said subject,
- (ii) exposing said isolated DNA to reactants and conditions for the amplification of a target region of the GST-Pi gene and/or its regulatory flanking sequences which includes a site or sites at which abnormal cytosine methylation characteristic of the disease or condition occurs, the amplification being selective in that it only amplifies the target region if the

said site or sites at which abnormal cytosine methylation occurs is/are methylated, and

(iii) determining the presence of amplified DNA,

wherein the amplifying step (ii) is used to amplify a target region of the GST-Pi gene and/or its regulatory flanking sequences defined by (and inclusive of)
5 CpG sites -43 to +55.

Since the amplification is designed to only amplify the target region if the said site or sites at which abnormal cytosine methylation (i.e. as compared to the corresponding site or sites of DNA from subjects without the
10 disease or condition being assayed) occurs is/are methylated, the presence of amplified DNA will be indicative of the disease or condition in the subject from which the isolated DNA has been obtained. The assay thereby provides a means for diagnosing or prognosing the disease or condition in a subject.

The step of isolating DNA may be conducted in accordance with
15 standard protocols. The DNA may be isolated from any suitable body sample, such as cells from tissue (fresh or fixed samples), blood (including serum and plasma), semen, urine, lymph or bone marrow. For some types of body samples, particularly fluid samples such as blood, semen, urine and lymph, it may be preferred to firstly subject the sample to a process to enrich
20 the concentration of a certain cell type (e.g. prostate cells). One suitable process for enrichment involves the separation of required cells through the use of cell-specific antibodies coupled to magnetic beads and a magnetic cell separation device.

Prior to the amplifying step, the isolated DNA is preferably treated
25 such that unmethylated cytosines are converted to uracil or another nucleotide capable of forming a base pair with adenine while methylated cytosines are unchanged or are converted to a nucleotide capable of forming a base pair with guanine. This treatment permits the design of primers which enable the selective amplification of the target region if the said site or
30 sites at which abnormal cytosine methylation occurs is/are methylated.

Preferably, following treatment and amplification of the isolated DNA, a test is performed to verify that unmethylated cytosines have been efficiently converted to uracil or another nucleotide capable of forming a base pair with adenine, and that methylated cytosines have remained unchanged or efficiently converted to another nucleotide capable of forming a base pair with guanine.

Preferably, the treatment of the isolated DNA involves reacting the isolated DNA with bisulphite in accordance with standard protocols. As will be clear from the above discussion of bisulphite treatment, unmethylated cytosines will be converted to uracil whereas methylated cytosines will be unchanged. Verification that unmethylated cytosines have been converted to uracil and that methylated cytosines have remained unchanged may be achieved by;

- (i) restricting an aliquot of the treated and amplified DNA with a suitable restriction enzyme(s) which recognise a restriction site(s) generated by or resistant to the bisulphite treatment, and
- (ii) assessing the restriction fragment pattern by electrophoresis.

Alternatively, verification may be achieved by differential hybridisation using specific oligonucleotides targeted to regions of the treated DNA where unmethylated cytosines would have been converted to uracil and methylated cytosines would have remained unchanged.

The amplifying step may involve polymerase chain reaction (PCR) amplification, ligase chain reaction amplification (20) and others (21).

Preferably, the amplifying step is conducted in accordance with standard protocols for PCR amplification, in which case, the reactants will typically be suitable primers, dNTPs and a thermostable DNA polymerase, and the conditions will be cycles of varying temperatures and durations to effect alternating denaturation of strand duplexes, annealing of primers (e.g. under high stringency conditions) and subsequent DNA synthesis.

To achieve selective PCR amplification with bisulphite-treated DNA, primers and conditions may be used to discriminate between a target region including a site or sites of abnormal cytosine methylation and a target region where there is no site or sites of abnormal cytosine methylation. Thus, for amplification only of a target region where the said site or sites at which abnormal cytosine methylation occurs is/are methylated, the primers used to anneal to the bisulphite-treated DNA (i.e. reverse primers) will include a guanine nucleotide(s) at a site(s) at which it will form a base pair with a methylated cytosine(s). Such primers will form a mismatch if the target region in the isolated DNA has unmethylated cytosine nucleotide(s) (which would have been converted to uracil by the bisulphite treatment) at the site or sites at which abnormal cytosine methylation occurs. The primers used for annealing to the opposite strand (i.e. the forward primers) will include a cytosine nucleotide(s) at any site(s) corresponding to site(s) of methylated cytosine in the bisulphite-treated DNA.

Preferably, the primers used for the PCR amplification are of 12 to 30 nucleotides in length and are designed to anneal to a sequence within the target region that includes two to four cytosine nucleotides that are abnormally methylated in the DNA of a subject with the disease or condition being assayed. In addition, the primers preferably include a terminal nucleotide that will form a base pair with a cytosine nucleotide (reverse primer), or the guanine nucleotide opposite (forward primer), that is abnormally methylated in the DNA of a subject with the disease or condition being assayed.

The step of amplifying is used to amplify a target region within the GST-Pi gene and/or its regulatory flanking sequences. The regulatory flanking sequences may be regarded as the flanking sequences 5' and 3' of the GST-Pi gene which include the elements that regulate, either alone or in combination with another like element, expression of the GST-Pi gene.

In particular, the step of amplifying is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +55 (wherein the numbering of the CpG sites is relative to the transcription start site). The numbering and position of CpG sites is shown in Figure 1.

The step of determining the presence of amplified DNA may be conducted in accordance with standard protocols. One convenient method involves visualisation of a band(s) corresponding to amplified DNA, following gel electrophoresis.

Preferably, the disease or condition to be assayed is selected from cancers, especially hormone dependent cancers such as prostate cancer, breast cancer and cervical cancer, and liver cancer.

For the diagnosis or prognosis of prostate cancer, the step of amplifying preferably amplifies a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +53, more preferably, -43 to +10. However, within these target regions it is believed that there are CpG sites which show variability in methylation status in prostate cancer or are methylated in other tissues. Thus, for the target region defined by (and inclusive of) CpG sites -43 to +10, it is preferred that the primers used for amplification be designed so as to minimise (i.e. by use of redundant primers or by avoidance of the sites) the influence of CpG sites -36, -32, -23, -20, -19, -14 and a polymorphic region covering site -33. Further, for DNA isolated from cells other than from prostate tissue (e.g. blood), it is preferred that the primers used be designated to amplify a target region that does not include the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -7 to +7, or, more preferably, -13 to +8, since this may lead to false positives. Further preferred target regions, therefore, are within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -14, -43 to -8, +9 to +53 and +1 to +53.

Suitable primer pairs for the diagnosis or prognosis of prostate cancer, include those consisting of a forward and reverse primer selected from each of the following groups:

Forward Primers (i.e. anneal to the 5' end of the target region)

- | | | |
|----|-------------------------------|----------------|
| 5 | CGCGAGGTTTTCGTTGGAGTTTCGTCGTC | (SEQ ID NO: 1) |
| | CGTTATTAGTGAGTACGCGCGGTTC | (SEQ ID NO: 2) |
| | YGGTTTTAGGGAATTTTTTTTCGC | (SEQ ID NO: 3) |
| | YGGYGYGTTAGTTYGTTGYGTATATTC | (SEQ ID NO: 4) |
| | GGGAATTTTTTTTCGCGATGTTYGGCGC | (SEQ ID NO: 5) |
| 10 | TTTTTAGGGGGTTYGGAGCGTTTC | (SEQ ID NO: 6) |
| | GGTAGGTTGYGTTTATCGC | (SEQ ID NO: 7) |

Reverse Primers (i.e. anneal to the extension of the forward primer)

- | | | |
|----|-----------------------------------|-----------------|
| | TCCCATCCCTCCCCGAAACGCTCCG | (SEQ ID NO: 8) |
| | GAAACGCTCCGAACCCCCTAAAAACCGCTAACG | (SEQ ID NO: 9) |
| 15 | CRCCCTAAAATCCCCRAAATCRCCGCG | (SEQ ID NO: 10) |
| | ACCCCRACRACCRCTACACCCRAACGTCG | (SEQ ID NO: 11) |
| | CTCTTCTAAAAAATCCCRCAACTCCCGCCG | (SEQ ID NO: 12) |
| | AAAACRCCCTAAAATCCCCGAAATCGCCG | (SEQ ID NO: 13) |
| | AACTCCCRCCGACCCCAACCCGACGACCG | (SEQ ID NO: 14) |
| 20 | AAAAATTCRAATCTCTCCGAATAAACG | (SEQ ID NO: 15) |
| | AAAAACCRAAATAAAAACCACACGACG | (SEQ ID NO: 16) |

wherein Y is C, T or, preferably, a mixture thereof, and R is A, G or, preferably, a mixture thereof.

- 25 For the diagnosis or prognosis of liver cancer, the step of amplifying preferably amplifies a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -14 and/or +9 to +53. However, within these target regions it is believed that there are CpG sites which show variability in methylation status in liver cancer or are methylated in other tissues. Thus, for the target region defined
- 30 by (and inclusive of) CpG sites -43 to -14, it is preferred that the primers used

for amplification be designed so as to minimise (i.e. by use of redundant primers or by avoidance of the sites) the influence of CpG sites -36, -32, -23, -20, -19, -14 and a polymorphic region covering site -33.

It will be appreciated by persons skilled in the art, that a site or sites of abnormal cytosine methylation within the above identified target regions of the GST-Pi gene and/or its regulatory flanking sequences, could be detected for the purposes of diagnosing or prognosing a disease or condition (particularly, prostate cancer and/or liver cancer) by methods which do not involve selective amplification. For instance, oligonucleotide/polynucleotide probes could be designed for use in hybridisation studies (e.g. Southern blotting) with bisulphite-treated DNA which, under appropriate conditions of stringency, selectively hybridise only to DNA which includes a site or sites of abnormal methylation of cytosine(s). Alternatively, an appropriately selected informative restriction enzyme(s) could be used to produce restriction fragment patterns that distinguish between DNA which does and does not include a site or sites of abnormal methylation of cytosine(s).

Thus, in a second aspect, the present invention provides a diagnostic or prognostic assay for a disease or condition in a subject said disease or condition characterised by abnormal methylation of cytosine at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its regulatory flanking sequences, wherein said assay comprises the steps of;

- (i) isolating DNA from said subject, and
- (ii) determining the presence of abnormal methylation of cytosine at a site or sites within the region of the GST-Pi gene and/or its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +55.

The step of isolating DNA may be conducted as described above in relation to the assay of the first aspect.

Preferably, the region of the GST-Pi gene and its regulatory flanking sequences within which the presence of methylated cytosine(s) at a site or sites is determined is selected from the regions defined by (and inclusive of)

CpG sites -43 to +53, -43 to +10, -43 to -14, +9 to +53 and +1 to +53. However, within these regions, it is preferred that certain sites (namely, CpG sites, -36, -33, -32, -23, -20, -19, and -14) be avoided as the site or sites at which, for the purpose of the assay, the presence of abnormal methylation of cytosine is determined.

Where the determination step is to involve selective hybridisation of oligonucleotide/polynucleotide/peptide-nucleic acid (PNA) probes, prior to the determination step, the isolated DNA is preferably treated (e.g. with bisulphite) such that unmethylated cytosines are converted to uracil or another nucleotide capable of forming a base pair with adenine while methylated cytosines are unchanged or are converted to a nucleotide capable of forming a base pair with guanine. This treatment permits the design of probes which allow for selective hybridisation to a target region including a site or sites of abnormal methylation of cytosine.

In a third aspect, the present invention provides a primer or probe (sequence shown in the 5' to 3' direction) comprising a nucleotide sequence selected from the group consisting of:

- | | | |
|----|----------------------------------|-----------------|
| | CGCGAGGTTTTCGTTGGAGTTTCGTCGTC | (SEQ ID NO: 1) |
| | CGTTATTAGTGAGTACGCGCGGTTC | (SEQ ID NO: 2) |
| 20 | YGGTTTTAGGGAATTTTTTTTCGC | (SEQ ID NO: 3) |
| | YGGYGYGTTAGTTYGTTGYGTATATTTTC | (SEQ ID NO: 4) |
| | GGGAATTTTTTTTCGCGATGTTTYGGCGC | (SEQ ID NO: 5) |
| | TTTTTAGGGGGTTYGGAGCGTTTC | (SEQ ID NO: 6) |
| | GGTAGGTTGYGTTTATCGC | (SEQ ID NO: 7) |
| 25 | AAAAATTCRAATCTCTCCGAATAAACG | (SEQ ID NO: 8) |
| | AAAAACCRAAATAAAAACCACACGACG | (SEQ ID NO: 9) |
| | TCCCATCCCTCCCCGAAACGCTCCG | (SEQ ID NO: 10) |
| | GAAACGCTCCGAACCCCTAAAAACCGCTAACG | (SEQ ID NO: 11) |
| | CRCCCTAAAATCCCCRAAATCRCCGCG | (SEQ ID NO: 12) |
| 30 | ACCCCRACRACCRCTACACCCCRACGTCG | (SEQ ID NO: 13) |

CTCTTCTAAAAAATCCCRCAACTCCCGCCG (SEQ ID NO: 14)

AAAACRCCCTAAAATCCCCGAAATCGCCG (SEQ ID NO: 15)

AACTCCCRCCGACCCCAACCCCGACGACCG, (SEQ ID NO: 16)

wherein Y is C, T or, preferably, a mixture thereof, and R is A, G or,

5 preferably, a mixture thereof.

The terms "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated component, feature or step or group of components, features or steps with or without the inclusion of a further component, feature or step or group of components. features or steps.

The invention will now be further described with reference to the accompanying figures and following, non-limiting examples.

Brief description of the accompanying figures:

15 *Figure 1* shows the organisation and nucleotide sequence of the human GST-Pi gene. CpG sites are numbered relative to the transcription start site. Nucleotide Sequence numbering is according to the GST-Pi gene sequence of Genbank Accession No. M24485.

20 *Figure 2* shows the region of the GST-Pi gene exhibiting differential methylation in prostate cancer. The figure further shows the sequence and derivation of primers for the upstream region (from CpG site -43 to +10) and the common polymorphism encompassing CpG site -33 (shown above the sequence (p)). Underneath the GST-Pi sequence is shown the sequence of the derived strand after conversion of cytosines to uracil. The derived strand

25 is shown either assuming all CpGs are methylated (B-M) or un-methylated (B-U). Below this is shown specific primers designed to selectively amplify the methylated sequence.

Figure 3 shows the methylation status of each CpG site in isolated DNAs;

A - for the core promoter region through to the 3' end of the GST-Pi gene for the LNCaP (LN) cell line, DU145 (DU) cell line, PC3 cell line, PC3-M cell line and PC3-MM cell line, for DNA isolated from normal tissue samples from prostate cancer patients (2AN, BN and CN), for prostate tumour tissue (BC, CC, DC, XC, WC and 2AC) and for normal prostate tissue (Pr) from a person without prostate cancer:

B - for the core promoter region and upstream sequences of the GST-Pi gene from normal prostate tissue (from a person without prostate cancer), from three prostate cancer samples (BC, CC and DC) and for a number of other normal tissues. Patients B and D were polymorphic at CpG site -33 and the level of methylation indicated in the brackets reflects methylation of the allele which contains the CpG. For CpG sites -28 to +10, the level of methylation was determined by direct sequence analysis of the population of PCR molecules (17). For the upstream CpG sites, -56 to -30, PCR products were cloned and a number of individual clones sequenced (number indicated in brackets below the sample name). For normal tissues the level of methylation at each site was determined as the fraction of all clones containing a C at that position. For the cancer samples BC, CC and DC, the level of methylation shown is that among the clones which showed DNA methylation in the region from CpG site -43 to -30 (about half of the clones in each case).

In both *A* and *B*, a blank box indicates that the site was not assayed, and a "B" indicates that the status of the site could not be determined (e.g. because of a sequence blockage or it was beyond the range of the sequencing run). The level of methylation detected at each site is shown, none (-), up to 25% (+), 26-50% (++), 51-75% (+++) and 76-100% (+++). The Gleason Grade of tumour samples is also shown.

Figure 4 provides the results of amplification of bisulphite treated DNAs from a variety of tissues;

A - panel A (region covering the transcription start site) used CGPS-1 and 3 as outer primers and CGPS-2 and 4 as inner primers, Panel B used the outer primer pair CGPS-5 and 8 which encompass the region from CpG site -39 to -16 for first round amplification, followed by a second round of amplification with the CGPS-6 and 7 primers, amplifying a 140 bp fragment covering CpG sites -36 to -23. The lanes are 1. Brain, 2. Lung, 3. Skeletal muscle, 4. Spleen, 5. Pancreas, 6. "Normal" Prostate Aged 85 y.o., 7. "Normal" Prostate Aged 62 y.o., 8. Heart, 9. Bone Marrow, 10. Blood-1, 11. Blood-2, 12. Blood-3, 13. Liver-1, 14. Liver-2;

B - used the same primer pairs as that of the amplification shown in Figure 4A Panel B. with DNA from 10 prostate cancer tissue samples (c) and matched normal (n) tissue samples from the same prostates (a positive control (+) LNCaP DNA and a negative control (-) is also shown). Underneath. is the Gleason grade and the level of methylation of samples seen with non-selective primers.

C - used the same primer pairs as that of the amplification shown in Figure 4A Panel B, with DNA from a range of healthy tissues, blood from prostate cancer patients and various cell lines. The lanes are: Panel A 1-10 blood samples from prostate cancer patients during radical prostatectomy; Panel B 1. normal prostate-1, 2. normal prostate-2, 3. normal prostate-3, 4. normal prostate-4, 5. normal prostate-5, 6. HPV transformed prostate cell line, 7. blood from prostate patient PA (PSA=1000), 8. blood from prostate patient PB (PSA=56), 9. blood from prostate patient PC (PSA=18); and Panel C 1. LNCaP cell line, 2. Du145 cell line, 3. PC-3 cell line, 4. PC-3M cell line, 5. PC-3MM cell line, 6. Hela cell line, 7. leukemic DNA, 8. HepG2 cell line, 9. human liver DNA, 10. white blood cells, 11. MRC-5 cell line.

Figure 5 provides the results of amplification of bisulphite treated DNAs from seminal fluid of prostate cancer patients (c) and from men with

no diagnosed prostate cancer (n), using the outer primer pair CGPS-5 and 8 and CGPS-6 and 7 as the inner primer pair. The lanes are L. LNCaP cell line (positive control), D. DU145 cell line, P. PC-3 cell line (negative controls), and M. molecular weight markers.

5 *Figure 6* shows the results of amplification of bisulphite treated DNAs, wherein the DNA has been isolated from prostate tissue slides that had been identified as either cancerous or diseased with benign hyperplasia (BPH). Selective PCR amplification was conducted using the outer primer pair CGPS-5 and 8 and the inner primer pair CGPS-11 and 12.

10 *Figure 7* shows the results of amplification of bisulphite treated DNAs, wherein the DNA has been isolated from prostate cancer cells enriched from blood samples using magnetic beads coated with an anti-epithelial antibody. Different numbers of LNCaP prostate cancer cells were added to the blood samples (7A) or blood with added LNCaP cells stored for different times at 15 4°C or room temperature prior to DNA isolation.

Figure 8 provides the results of amplification of bisulphite treated DNAs. wherein the DNA has been isolated from blood samples from normal subjects with no known prostate complaint, from patients with benign hyperplasia (BPH) of the prostate and from patients with histologically 20 confirmed prostate cancer.

Figure 9 shows the results of amplification of bisulphite treated DNAs isolated from 20 liver cancer tissue samples. Selective PCR amplification was conducted using the outer primer pair CGPS-5 and 8 and the inner primer pair CGPS-11 and 12.

25 *Figure 10* shows the results of tests conducted to confirm that any amplified DNA products has occurred from amplification of bisulphite treated DNA wherein all unmethylated cytosine has been converted to uracil. The tests are conducted using oligonucleotides probes designed to hybridise to converted or non-converted target regions.

GENERAL METHODS AND STRATEGIES

(1) Treatment of DNA with bisulphite

DNA for assaying was isolated from suitable sources by standard protocols and treated with bisulphite by well known methods (12, 13, 16).

(2) Characterisation of Methylation of Individual Sites in DNA

In order to determine the methylation status of individual cytosine nucleotides in target and non-target DNAs and to identify differences between them, bisulphite-modified DNA was amplified by PCR using primers designed to minimise the possibility that the methylation status of a particular CpG site will influence primer annealing and subsequent amplification (12, 13, 16).

(3) Design of Selective Primers

Based on the sequencing information, primers for use in the assay were designed to maximise the possibility that the methylation status of a particular CpG site would influence primer annealing and subsequent amplification. Specifically, the design principles followed (described for the "forward" PCR primer where the primer contains the same C to T (or U) conversions as would occur in the bisulphite-treated DNA), are listed below at (a) to (d):

- (a) That primers should cover sequence regions which contain a number of C's. Conversion of unmethylated C's to U's provides for discrimination between molecules which have undergone efficient bisulphite conversion and molecules in which C's have not reacted (e.g. because not completely dissolved or containing regions of secondary structure).
- (b) That at least one, but preferably at least two to four, of the C's in the regions should be C's (generally at CpG sites) known to be methylated in a high proportion of the DNA to be detected (i.e. target DNA). Thus, these C's will remain C's in the target DNA while being converted to U's in the non-target DNA. A primer which is designed to be the exactly equivalent of the bisulphite-converted methylated DNA will contain a mismatch at each of the

positions of an unmethylated C which has been converted to a U in an unmethylated DNA. The more mismatches that are present, the greater the differential hybridisation stability of the primers will be and hence the greater the selective difference in PCR.

- 5 (c) That the 3' terminal base of the primer should preferably be a C corresponding to a C known to be methylated in the target DNA (normally part of a CpG dinucleotide). Correct pairing with the terminal base of the primer will provide for highly selective priming of target sequences compared with unmethylated background sequences which will form a C:A mismatch.
- 10 (d) That at positions where it is known that methylation occurs in only a fraction of molecules in the methylated target DNA or where it is known to vary between target DNAs (e.g. in different tumour samples), redundancy can be incorporated into the primers to allow for amplification of either C or T
- 15 from the target DNA. This same approach can be used if polymorphisms are known to exist in the primer region.

For the "reverse" primer, which anneals to the converted strand, A's replace G's at positions opposite converted C's.

(4) *Verification of Selective Target Sequence Amplification*

20 The amplified PCR band can be analysed to verify that it has been derived from DNA which has been fully bisulphite-converted (i.e. C's not methylated in the original DNA have been converted to U's and amplified as T's) and to further verify that the amplified DNA has been derived from the specific target DNA sequence and has the expected methylation profile (i.e. 5meC's

25 not converted to T's). Methods for conducting these verifications include:

- (a) Using restriction enzyme digestion.

In order to verify complete conversion, particular restriction enzymes can be used to cut the DNA. The sequence recognition sites should have the property that they contain no C's and are present in the sequence of the

30 amplified strand after but not before bisulphite treatment. Thus, the

conversion of one or preferably two or more C's to U's and their amplification as T's in the PCR product should produce a new restriction site. Useful enzymes are shown in *italics* in Table 1 below.

In order to verify that the target DNA sequence amplified was specifically methylated, use can be made of restriction enzyme sites whose only C nucleotides are found as CpG dinucleotides and which, if the sequence was methylated, would remain as CpG's in the PCR products. Examples of such enzymes are shown in **bold** in Table 1 below. *BsmBI*, which cuts the non-symmetrical sequence GAGACG can also be used.

In some instances, enzymes which contain a C as an outer base in their recognition sequence can be used for verification of methylation: e.g. *EcoRI* (GAATTC) for a GAATTCG sequence or *Sau3AI* (GATC) for a GATCG sequence (**bold and underlined** in Table 1). If a site such as one of the above is present in the predicted methylated, fully bisulphite-converted DNA then the enzyme will cut the DNA only if the original CpG dinucleotide was methylated, confirming the amplification of a methylated region of DNA. Some of the enzymes (**bold and underlined** in Table 1) have the potential to be used both for monitoring efficient conversion and CpG methylation.

(b) Differential hybridisation to specific oligonucleotides.

Differential hybridisation to specific oligonucleotides can be used to discriminate that the amplified DNA is fully reacted with bisulphite and of the expected methylation profile. To demonstrate complete conversion, a pair of oligonucleotides corresponding to the same region within the amplified sequence is prepared. One oligonucleotide contains T's at all C's which should be converted by bisulphite, while the other contains C's in these positions. The oligonucleotides should contain at least two or three of such discriminatory C's and conditions be determined which provide for selective hybridisation of each to its target sequence. Similar oligonucleotides with C or T at CpG sites and T's replacing all non-CpG C's are used to determine whether the specific CpG sites are methylated.

Additional control oligonucleotides that contain no discriminatory C's, that is, either no C's or a minimal number where C's are substituted with Y's (mixture of C and T), are used to monitor the amount of PCR product in the sample. The oligonucleotides can be used for direct hybridisation detection of amplified sequences or used to select out target molecules from the PCR-amplified DNA population for other detection methods. An array of such oligonucleotides on a DNA sequencing chip can be used to establish the sequence of the amplified DNA throughout the sequence region.

(c) Single nucleotide primer extension (SNUPE).

The technique of single nucleotide primer extension can be applied to the PCR products to determine whether specific sites within the amplified sequence contain C or T bases. In this method, a primer abutting the position of interest is annealed to the PCR product and primer extension reactions performed using either just dCTP or just dTTP. The products can be separated by gel electrophoresis and quantitated to determine the proportion of each nucleotide in the population at that position. Primers should be designed to quantitate conversion of C's in CpG sites and control C's which should not be methylated. More than one primer can be included in a single reaction and/or run in the same gel track as long as their sizes can be clearly distinguished.

(d) Fluorescent Real-time Monitoring of PCR.

Oligonucleotides internal to the amplified region can be used to monitor and quantify the amplification reaction at the same time as demonstrating amplification of the correct sequence. In the Fluorogenic 5' Nuclease PCR assay (19) the amplification reaction is monitored using a primer which binds internally within the amplified sequence and which contains both a fluorogenic reporter and a quencher. When this probe is bound to its target DNA it can be cleaved by the 5' nuclease activity of the Taq polymerase, separating the reporter and the quencher. By utilising in the assay an oligonucleotide which is selective for the fully bisulphite-converted

sequence (and/or its methylation state) both the level of amplification and its specificity can be monitored in a single reaction. Other related systems that similarly detect PCR products by hybridisation can also be used.

5 **Example 1: Methylation sequence profile of target and non-target GST-Pi**
 DNA

MATERIALS AND METHODS

 Figure 1 shows the organisation of the GST-Pi gene and the regions for
 which genomic sequencing was used to determine the methylation status of
10 DNA isolated from prostate cancer tissue or cell lines and from normal
 prostate or other tissues. The nucleotide sequence numbering in Figure 1 is
 according to the GST-Pi sequence, Genbank Accession No. M24485. Also
 shown, within the boxes is the sequence of each amplified region, with all
 the CpG sites indicated and numbered relative to the position of the
15 transcription start site. Sequence analysis demonstrated that there was an
 additional CpG dinucleotide (+9) not predicted from the published sequence.
 Also identified in the regions sequenced was a polymorphism which is
 present in a significant fraction of the samples studied. The polymorphic
 allele does not contain CpG site -33. Both the additional CpG dinucleotide
20 and the polymorphism are shown in Figure 2. The nucleotide coordinates in
 Figure 2 are shown relative to the transcription start site; the first base
 shown, -434, corresponds to base 781 of the Genbank sequence, while the
 last +90, corresponds to base 1313 of the Genbank sequence.

 Table 2 lists the sequences and positions of the non-selective primers
25 used for amplification (Table 2-1) and direct sequencing (Table 2-2) of
 bisulphite-treated DNA.

 DNA isolated from normal prostate tissue, prostate cancer tissue,
 prostate cancer-derived cell lines and other tissues was bisulphite treated and
 PCR reactions done by standard procedures (13). PCR products were either

digested with informative restriction enzymes, sequenced directly (17), or individual molecules cloned and sequenced by standard procedures.

RESULTS

In Figure 3A, the methylation status of sites in DNA from prostate cancer cell lines, prostate cancer tissue samples and matched normal prostate tissue are shown for the core promoter regions through to the 3' end of the gene (covering CpG sites -28 to 103). It can be seen that in normal prostate tissue, the core promoter region is unmethylated at all sites and that this lack of methylation extends through the region flanking the promoter to CpG site +33. Results of restriction enzyme digests of bisulphite-treated, PCR-amplified DNA indicate that this lack of methylation includes CpG sites +52 and +53. However, in the regions further downstream which were analysed, CpG sites +68 to +74 and +96 to +103, DNA from normal prostate tissue was heavily methylated. Analysis of the prostate cancer cell line LNCaP and prostate cancer tissue samples demonstrates extensive methylation of the core promoter region; variations in the overall level of methylation probably reflect the presence of different levels of normal cells within the tumour samples. DNA from one cancer sample (2AC) was found to be completely unmethylated and in contrast to the other tumour samples this tumour was found by immunohistochemistry to still be expressing GST-Pi. Sequencing of the region flanking the core promoter in the LNCaP cell line and tumour DNAs, BC and CC, showed that methylation extended through to CpG site +33 and further restriction enzyme analysis showed that methylation included CpG sites +52 and +53. For one tumour sample, DC, methylation did not extend beyond the core promoter region and CpG sites +13 to +33, as well as CpG sites +52 and +53 were found to be unmethylated. It is notable that this tumour was of Gleason Grade 2+2, the lowest grade tumour among those analysed. For all tumour DNA samples, as for the normal DNA, the downstream regions of the gene, sites 68 to 74 and 96 to 103, were heavily methylated. Within the promoter regions which were methylated in

the cancer, but not normal, tissue specific individual sites were evident which were either unmethylated or methylated to a much lower degree than surrounding methylated sites. These include sites -22 and -23 (XC), -20 (PC3 lines. XC and WC), -14 (PC3, XC and WC), +24 (PC3-M and MM2, CC), +25 (LNCaP, PC3-MM2, CC).

The results shown in Figure 3B provides a comparison of the methylation state of the core promoter region and sequences upstream of the core promoter region in DNA isolated from normal prostate tissue and from a number of other normal tissues. Sequences from the PCR fragment upstream of the core promoter were determined by cloning and sequencing as the region is refractory to direct sequencing. For the cancer samples, the level of methylation shown is as a proportion of those clones which were methylated (about 50% of the total clones in both cases). In normal prostate tissue as well as in all other normal tissues there is extensive methylation of CpG sites upstream of the AT-rich repeat. Downstream of the repeat (from CpG site -43) minimal methylation was seen in all normal tissues except normal liver tissue, where there was significant methylation of CpG sites -7 through to +7. Sequences upstream of the core promoter were found to be heavily methylated in the prostate cancer DNAs, though again specific sites were undermethylated; site -32 in cancers B and D and site -36 in cancer B.

The results therefore allow for the identification of a region of the GST-Pi gene and its regulatory flanking sequences, stretching from 3' of the polymorphic repeat region, (CpG site -43) to sites +52 and +53, which is not methylated in normal prostate tissue but is normally highly methylated in prostate cancer. In one cancer sample (D, the cancer of lowest Gleason Grade) the region from CpG sites +13 to +53 was not methylated. The more restricted region extending from CpG site -43 to +10 was methylated in all of the prostate cancer DNAs which showed promoter methylation. Methylation of part of the promoter region (CpG sites -7 to +7) was also seen in one normal tissue (liver) examined. Analysis of further samples of normal liver

DNA has shown that the level of methylation is variable and can include CpG sites from -13 to +8.

DISCUSSION

The above results are critical in identifying regions within the GST-Pi gene and/or its regulatory flanking sequences which can be used for the development of assays for the selective detection of prostate cancer cells. Thus, the region from CpG sites -43 to +53 lying within the boundary of regions methylated in normal prostate tissue can be used for the design of primers to detect cancer-specific methylation in prostate tissue samples. The region from CpG site -43 to +10 is preferred for the detection of a higher proportion of cancers. The region from CpG sites +13 to +53 may be used to detect cancer but also may be used to distinguish early (unmethylated) cancer from later (methylated cancer). For assays using other samples, such as blood, it is preferred to restrict the region chosen to exclude CpG sites -7 to +7 or, more preferably sites -13 to +8. For example, liver cells may be present in the blood taken from a subject suffering liver disease, in which case, a false positive result could be obtained if the region chosen for detection of cancer-specific methylation includes CpG sites -13 to +8.

Example 2: Design and use of selective primers for detection of methylated GST-Pi DNA

MATERIALS AND METHODS

Sequence primers for the detection of methylated GST-Pi sequences from three regions, namely a region upstream of the core promoter (primers CGPS-5 to 9 and CGPS-11 to 13), a region partially encompassing the core promoter (primers CGPS-1 to 4), and a region further downstream from the core promoter (primers CGPS-21 to 24) are shown in Table 3 below.

The sequence and derivation of primers for the upstream region are shown in Figure 2 (from CpG site -43 to CpG site +10), which also shows the common polymorphism encompassing CpG site -33 (see above the sequence

(p)). Underneath is shown the sequence of the derived strand after conversion of cytosines to uracil. The derived strand is shown either assuming all CpGs are methylated (B-M) or that none are (B-U). Below this is shown specific primers designed to selectively amplify the methylated sequence. It can be seen that all primers are designed to match perfectly to the treated, methylated template, but contain mismatches to the template derived from unmethylated DNA or the original untreated DNA. Primers CGPS-5, 8, 11, 12 and 13 are designed to avoid the polymorphic region and CpG sites which show a lower frequency of methylation in prostate cancer DNAs. The underlined T's in the forward primers (and A's in the reverse primers) derive from bisulphite conversion of C's and provide discrimination against amplification of DNA which has not been efficiently converted by the bisulphite treatment. The bold C's in the forward primers (and G's in the reverse primers) are parts of CpG sites and will form base pairs with DNA derived from methylated sequences but form mismatches to DNA derived from unmethylated sequences. Redundancy is included in some positions, Y (= mix of C and T) in forward primers and R (= mix of A and G) in reverse primers to allow pairing independent of methylation status. This can allow for certain sites where the frequency of methylation within or between tumour samples is variable (eg. site -14). Forward and reverse primers for specific selective amplification of methylated GST-Pi sequences are shown in Table 3 below.

Amplifications conducted for this example, utilised bisulphite treated DNAs from a variety of tissues and used two sets of PCR primers.

Specifically, for the amplification reactions shown in Figure 4A Panel A (region covering the transcription start site), CGPS-1 and 3 were used as outer primers and CGPS-2 and 4 as inner primers. For the amplification reactions shown in Figure 4A Panel B and Figure 4B and 4C, the outer primer pair, CGPS-5 and CGPS-8 which encompass the region from CpG site

-39 to -16, were used for first round amplification, followed by second round amplification with the CGPS-6 and CGPS-7 primers, resulting in the amplification of a 140 bp fragment covering CpG sites -36 to -23. For the amplification reactions shown in Figures 5 to 8, the primer set used for the upstream region was the outer primer pair, CGPS-5 and CGPS-8, for first round amplification and the inner primer pair, CGPS-11 and CGPS-12, for second round amplification, resulting in the amplification of a 167 bp fragment covering CpG sites -38 to -23.

For all sets of primers, PCR amplifications were performed in a buffer consisting of 67 mM Tris/HCl, 16.6 mM ammonium sulphate, 1.7 mg/ml BSA and 1.5 mM MgCl₂, prepared in TE buffer (10 mM Tris/HCl pH 8.8, 0.1 mM EDTA). Reaction mixes (50 µl) contained 200 µM of each of the four dNTPs, 6 ng/ml of each primer and 2 units of AmpliTaq DNA polymerase (Perkin Elmer). For the primers CGPS-5 and 8 (first round amplification), PCR cycle conditions were 5 cycles of 60°C 1 min., 72°C 2 min. and 95°C 1 min., followed by 30 cycles of 65°C 1 min., 72°C 1.5 min. and 95°C 1 min. Amplification conditions for the primers CGPS-6 and 7 (second round amplification) were 5 cycles of 65°C 1 min., 72°C 2 min. and 95°C 1 min., followed by 30 cycles of 65°C 1 min., 72°C 1.5 min. and 95°C 1 min. For the primers CGPS-11 and 12, the amplification conditions were the same as for the CGPS-6 and 7 primers except that the annealing temperature was raised from 65°C to 70°C. 2 µl of the first round amplification reactions were used in 50 µl of second round amplification reactions. Other buffers or PCR amplification conditions may also be used to achieve similar efficiency and specificity.

RESULTS AND DISCUSSION

For the primers covering the core promoter region (see Figure 4A Panel A), amplified DNA (see arrowed band) was obtained from the positive control DNA (cancer B) but also from DNA from prostate tissue samples from two subjects who had not been diagnosed with prostate cancer. Bands of

amplified DNA were also seen from DNA isolated from a bone marrow and blood sample as well as from DNA isolated from liver tissue samples from subjects with no known prostate cancer.

For the upstream amplification (see Figure 4A Panel B), no amplified DNA was obtained from amplification reactions conducted on DNA isolated from a range of healthy tissue samples nor from DNA isolated from blood samples of subjects with no known prostate cancer; a band of amplified DNA was produced from the positive control DNA (cancer B). However, while amplification reactions conducted on DNA isolated from one normal prostate tissue sample did not result in amplified DNA, amplified DNA did result from the same amplification reactions conducted on DNA isolated from a prostate tissue sample of an 82 year old subject with no known prostate cancer. It is possible that this subject had undiagnosed prostate cancer. DNA isolated from five other samples of normal prostate tissue from subjects with no known prostate cancer did not give rise to an amplified DNA product (see Figure 4C Panel B).

In Figure 4B, the results of PCR amplification reactions are shown for tissue samples from patients with prostate cancer: for each sample, DNA was isolated from a region identified as containing cancer and from another region identified as grossly normal. In all cases, a clear band of amplified DNA was produced from amplification reactions conducted on prostate cancer DNA. Two of these, were cases where the proportion of methylated DNA was insufficient to be detected using primers designed to prime equivalently on methylated and unmethylated DNA. For DNA isolated from grossly normal tissue, the band of amplified DNA was either absent or present in a substantially lower amount. The presence of a band in some "normal" samples could derive from a low level of cancer cells in the sample.

Amplification of DNA from samples of blood obtained from the abdominal cavity during surgery showed that it was possible to detect methylated GST-Pi sequences in a number of them. Samples of peripheral

blood isolated from three patients with known metastatic disease (see Fig 4C Panel B) demonstrated the presence of amplifiable, methylated GST-Pi sequences.

Amplified DNA products were also produced from amplification of DNA isolated from the LNCaP and DU145 prostate cancer cell lines, but not from the PC-3 series of cell lines. This latter result could be due to a low level of methylation in the upstream promoter region in PC-3 cells, but a major contributing factor is likely to be a lack of priming by the CGPS-6 primer as PC-3 only contains the variant allele of the GST-Pi gene.

Methylated GST-Pi sequences were also detected in DNA isolated from some tumour-derived cell lines of non-prostatic origin: HeLa, a cervical carcinoma, and HepG2, a liver carcinoma (see Figure 4C Panel B).

DNA was isolated from the seminal fluid (see Figure 5) of 3 prostate cancer patients (C) and from 5 subjects with no known prostate cancer (N), treated with bisulphite and amplified using primers CGPS-5 and 8 followed by CGPS-6 and 7. Amplified DNA products were obtained from all three cancer DNAs. One of the five samples from subjects without diagnosed prostate cancer also resulted in an amplified DNA product, but it is not clear if this represents a false positive or a case of undiagnosed prostate cancer in the particular subject.

The use of the primer CGPS-11 avoids annealing across the polymorphic sequence at CpG site -33, and the combination of CGPS-5 and 8 as outer primers followed by CGPS-11 and 12 as inner primers was found to give efficient amplification of prostate cancer DNA. In a first experiment (see Figure 6), DNA was extracted from regions of fixed tissue slides that had been identified as either being cancerous or being diseased with benign hyperplasia (BPH). DNA was isolated by incubating scraped material in 400 µl of 7M guanidinium hydrochloride, 5 mM EDTA, 100 mM Tris/HCl pH 6.4, 1% Triton-X100, 50 mg/ml proteinase K and 100 mg/ml yeast tRNA. After homogenisation, samples were incubated for 48 hours at 55°C then subjected

to five freeze/thaw cycles of dry ice for 5 min./95°C for 5 min. After vortexing and centrifugation for 2 min. in a microfuge, the supernatants were then diluted three fold, extracted with phenol/chloroform and ethanol precipitated. DNA isolated from samples from 6 cancer patients and 4 with BPH were amplified with either non-selective primers for the core promoter region (i.e. control PCR amplification with GST-9 and 10 followed by GST-11 and 12) or CG selective primers (i.e. selective PCR amplification with CGPS-5 and 8 followed by CGPS-11 and 12). Control PCR amplifications demonstrated the presence of amplifiable DNA in all samples. Using the CG selective primers, amplified DNA products were only obtained from the cancer DNAs. The PSA (prostate specific antigen) levels of these patients ranged from 4 to 145 ng/ml. For the BPH patients, the PSA levels ranged from 2.3 to 25 ng/ml.

In further experiments, prostate cancer cells were first enriched from blood samples using antibodies coupled to magnetic beads followed by DNA isolation, bisulphite modification and PCR amplification. Cell isolation was achieved using Dynabeads anti-Epithelial Cell (Dynal Prod. No. 112.07) essentially as described by the manufacturer. The magnetic beads were coated with the anti-epithelial antibody mAb Ber-EP4 (22). Alternatively, magnetic beads coupled to antibodies specific for the extracellular domain of the prostate specific membrane antigen (23) could have been used. Whole blood was diluted 1:1 with Dulbecco's phosphate buffered saline (PBS) containing 10 mM EDTA and 40 µl of pre-washed magnetic beads added. Cells were incubated at 4°C on a rotating platform for 30 min and then the beads were collected to the side of the tube using a magnetic cell separation device for 4 min. The supernatant was then carefully aspirated and the beads resuspended in the washing solution (PBS containing 0.5% bovine serum albumin). Beads were then again collected to the side of the tube using a magnet and the supernatant carefully aspirated before conducting a further wash was done with the tube remaining in place in the magnetic

separation device and the supernatant aspirated. The beads were then resuspended in DNA isolation buffer (100 mM Tris/HCl pH 8, 25 mM EDTA, 1% Sarkosyl, 200 mg/ml proteinase K), incubated for at least 2 h at 37° and DNA recovered by phenol/chloroform extraction and ethanol precipitation.

5 The DNA was then finally subjected to bisulphite treatment and PCR amplification.

The sensitivity of this method was tested by seeding varying numbers of cells of a prostate cancer cell line, LNCaP, into normal blood. As shown in Figure 7A, the presence of 20 cells or more in 0.5 ml of blood could be reliably detected. The experiment shown in Figure 7B showed that blood samples containing LNCaP cells could be stored at room temperature or at 4 °C for up to 24 hours without loss of sensitivity.

Using magnetic bead capture followed by bisulphite treatment and selective PCR amplification, patient blood samples were also analysed and the results from a set of these are shown in Figure 8. These include blood samples from normal subjects with no known prostate complaint, from patients with benign hyperplasia (BPH) of the prostate and from patients with histologically confirmed prostate cancer. The control PCR amplifications (upper panel) used primers which amplify both methylated and unmethylated GST-Pi sequences. The amplifications using CG-selective primers are shown in the lower panel. Positive control amplifications (LNCaP (L) and PC3 (P)) are shown in the cancer panels and negative control amplifications are shown in the normal and cancer panels.

Table 4 below summarises the results of testing of DNA from patient blood samples using the magnetic bead/CG selective PCR amplification protocol. No amplified DNA products were obtained from DNA isolated from normal control subjects, and only DNA isolated from one of 18 patients diagnosed histologically to have BPH produced amplified DNA products (this patient had a blood PSA level of 17 ng/ml). Of patients with confirmed prostate cancer, isolated DNA from 17 of 24 (70%) were PCR-positive (i.e.

resulted in the production of amplified DNA), indicating the presence of prostate cancer cells in the blood. For patients clinically staged as A and B, (i.e. disease confined to the prostate), cancer cells were detected in the blood in 6 of of the 10 cases. For 9 patients with locally invasive (Stage C) or
5 metastatic (Stage D) disease, cancer cells were detected in the blood in every case.

Since it was found that the HepG2 liver cancer cell line contained methylated GST-Pi sequences, samples of DNA isolated from liver cancer tissue was also examined. DNA isolated from 20 liver cancer samples were
10 bisulphite treated and amplified using the CGPS-5 and 8 and CGPS-11 and 12 primer pairs (see Figure 9). 14 of the 20 samples were PCR-positive. On the other hand, no amplified DNA products were produced from DNA isolated from 2 patients with no liver cancer (see Figure 4 and data not shown). DNA isolated from normal liver tissue was shown to be partially methylated in the
15 region of the transcription start site (CpG sites -7 to + 7, see Figure 3B). Analysis of further samples of normal liver DNA has shown that the level of methylation is variable and can include CpG sites from -13 to +8. The primer pairs used here encompass CpG sites -39 to -16, upstream of the region of methylation seen in normal liver DNA.

20 The above results show that different sets of primers designed to hybridise the core promoter of the GST-Pi gene or the region upstream of the core promoter, can reliably amplify bisulphite-treated DNA that has been isolated from prostate cancer cells. However, primers designed to hybridise to the core promoter are less selective in that DNAs isolated from a number
25 of normal tissue samples result in amplified DNA products. Thus, primers designed to hybridise to regions found to be unmethylated in DNA from normal tissues, that is, the upstream region encompassing CpG sites -45 to -8 and the region downstream of the promoter encompassing CpG sites +8 to +53, are preferred for the prognostic or diagnostic assaying of prostate

cancer. Additionally, primers designed to hybridise to this latter region may also be useful for discriminating between early and late prostate cancer.

Example 3: Confirmation of correct amplification

5 The specific oligonucleotides probes described below can be used to confirm that any amplified DNA products resulting from the amplification step of the assay is due to DNA in which all unmethylated cytosines had been converted to uracils. Those for the upstream PCR region can be used with amplified DNA products from all combinations of the CGPS-5, 6, 11, 7
10 to 9, 12 and 13 forward and reverse primers. Those for the downstream PCR region can be used with amplified DNA products of the CGPS-21 to 24 primers. A biotinylated version of the conversion-specific oligonucleotide can also be used for the selective and specific capture from solution of the amplified DNA products generated using these primer pairs, or the
15 appropriately labelled oligonucleotide can be use for real-time monitoring of specific PCR fragment amplification. Amplified DNA products from PCR amplification of bisulphite-treated DNA routinely have one strand containing a very high proportion of thymine nucleotides and the other strand containing a very high proportion of adenine nucleotides. Because of this, it
20 is possible to use oligo dT (or oligo dA) as a generic conversion specific oligonucleotide, the annealing conditions being varied to optimise discrimination of converted and non-converted DNA for each PCR fragment.

Upstream PCR region:

25 Conversion oligonucleotide:

 HybC5 5'-AAACCTAAAAAATAACAAACAA (SEQ ID NO: 17)

 Non-conversion oligonucleotide:

 HybU5 5'-GGGCCTAGGGAGTAAACAGACAG (SEQ ID NO: 18)

 Conversion neutral oligonucleotide:

30 HybN5: 5'-CCTTTCCCTCTTTCCCARRTCCCCA (SEQ ID NO: 19)

Downstream PCR region:

Conversion oligonucleotide:

HyBC3 5'-TTTGGTATTTTTTTTCGGGTTTTAG (SEQ ID NO: 20)

5 Non-conversion oligonucleotide:

HybU3 5'-CTTGGCATCCTCCCCCGGGCTCCAG (SEQ ID NO: 21)

Conversion neutral oligonucleotide:

HybN3 5'-GGYAGGGAAGGGAGGYAGGGGYTGGG (SEQ ID NO: 22)

10 To demonstrate the selectivity of such hybridisations, a series of DNAs were spotted onto nylon membranes and hybridised with conversion and non-conversion specific oligonucleotide probes for the upstream PCR region as well as a control oligonucleotide. The DNAs included:

15 (i) individual cloned PCR products from amplification of the upstream region that contained differing numbers of converted cytosines in the region complementary to the probe (see Figure 10, where the number of converted cytosines, out of 10, is shown (Column 1 and top 2 spots of Column 2). n.b. the two clones containing 10/10 converted bases end adjacent to and do not contain the sequences complementary to the control oligonucleotide); and
20 (ii) PCR products from cancer patients and patients with benign hyperplasia that had been amplified from bisulphite-treated DNA using CG-selective primers (CGPS-5 and 8, followed by CGPS 11 and 12) (see figure 10, where these are labelled as Cancer Samples 1 to 4 (lower part of column 2) and BPH samples 1 to 4 (Column 3)).

25 Hybridisations with kinased oligonucleotide probes were performed in Express-Hyb buffer (Clontech) at 45°C for two hours followed by four 20 min. washes in 2X SSC, 0.1% SDS at 45°C before phosphorimage analysis.

Hybridisations with the control oligonucleotide probes provides an estimate of the amount of DNA in the sample. As expected, none of the PCR

amplifications of BPH samples produced significantly detectable product, while 3 of 4 cancer samples gave a strong signal and one a very weak one.

Hybridisations with the conversion-specific probe showed a clear signal for the plasmid DNAs that matched the probe perfectly and for the 3 cancer samples for which there was stronger hybridisation with the control oligonucleotide probe. The fourth cancer sample that gave a very weak signal with the control oligonucleotide was barely detectable with the conversion-specific probe. This could have been due to the low level of DNA or, possibly, the presence of partially-converted DNA molecules. None of the plasmid clones that had mismatches to the conversion-specific probe gave a significant signal. The probe for unconverted DNA hybridised clearly with plasmid DNAs that had 0, 1 or 2 bases converted, but not with samples that had 8 or 10 converted bases. The hybridisations also indicated that there was a low level amplification of unconverted DNA in two BPH and one cancer sample (in this latter case there was a strong signal from probe for fully converted DNA, indicating that the PCR product was predominantly derived from properly converted DNA).

The results show that oligonucleotides of the type used here can discriminate between molecules that have been efficiently converted by bisulphite and those that have not. They can be used in a number of formats for detection of PCR products or prior to PCR or other detection methods to select out efficiently converted molecules of the target region from the total DNA population. The same approach can be used with primers that distinguish CpG methylated DNAs (or their derivatives containing C's) from unmethylated DNAs (containing U's or their derivatives containing T's).

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TABLE 2.1 Primers for PCR Amplification of the Bisulphite-Modified GST-Pi Gene

PCR #	Target	Primer Name	Primer Type	Primer 5' 3'	Target size (bp)	Anneal °C	Genomic Position
-1	Upstream Top Strand DNA	GST-1	Outer	TTATGTAATAAAATTTGTATATTTTGTATATG (SEQ ID NO: 23)	646	50/50	381-411
		GST-25	Inner	TGTAGATTATTTAAGTTAGGAGTT (SEQ ID NO: 24)	499	50/50	495-519
		GST-3	Inner	AAACCTAAAAAATAAACAAACAACAAAA (SEQ ID NO: 25)	499	50/50	967-993
		GST-4	Outer	AAAAAACCTTTCCCTCTTTCCCAAAATCCC (SEQ ID NO: 26)	646	50/50	999-1027
1	Exon 1 Top Strand DNA	GST-9	Outer	TTTGTGTTTGTGTTTATTTTATAGTTT (SEQ ID NO: 27)	346	45/50	967-993
		GST-11	Inner	GGGATTTGGGAAAGAGGGAAAGGTTT (SEQ ID NO: 28)	307	45/50	999-1025
		GST-12	Inner	ACTAAAAAGCTCTAAACCCCATCCC (SEQ ID NO: 29)	307	45/50	1280-1303
		GST-10	Outer	AACCTAATACTACC TTAACCCCAT (SEQ ID NO: 30)	346	45/50	1304-1329

TABLE 2.1 continued

2	Exon 1 Bottom Strand DNA	GST-B1	Outer	AATCCTCTTCCTACTATCTATTTACTCCCTAAA (SEQ ID NO: 31)	387	50/55	958-990
		GST-B2	Inner	AAAACCTAAAAAATAAAAAAACITCCC (SEQ ID NO: 32)	314	50/55	999-1027
		GST-B3	Inner	TTGGTTTTATGTTGGGAGTTTGTGAGTTTT (SEQ ID NO: 33)	314	50/55	1285-1313
		GST-B4	Outer	TTTTGTGGGAGTTGGGGTTTGATGTTGT (SEQ ID NO: 34)	387	50/55	1317-1345
3	Exon 2/Exon 3 Top Strand DNA	GST-13	Outer	GGTTTAGAGTTTTTTAGTATGGGGTTAATT (SEQ ID NO: 35)	691	45/50	1287-1315
		GST-14	Inner	TAGTATTAGGTTAGGGTTTT (SEQ ID NO: 36)	603	45/50	1318-1337
		GST-15	Inner	AACTTAACCCTAATCTACCAACAACATA (SEQ ID NO: 37)	603	45/50	1920-1892
		GST-16	Outer	CA AAAAACITTTAAATAAACCCCTCCTACCA (SEQ ID NO: 38)	691	45/50	1978-1950

002277 84462960

TABLE 2.1 continued

4	Exon 5 Top Strand DNA	GST-30	Outer	GTTTTGTTAGTTGTTTTTTAGGTGTAG (SEQ ID NO: 39)	340	55/60	2346-2376
		GST-31	Inner	GTTTTGAGTATTGTTGTTGTTGTTGTTT (SEQ ID NO: 40)	265	40/45	2381-2416
		GST-32	Inner	TTAATATAAATAAAAAAATATATTACAA (SEQ ID NO: 41)	265	40/45	2617-2646
		GST-33	Outer	CAACCCCAATACCCAACCCCTAATACAAATCTC (SEQ ID NO: 42)	340	55/60	2653-2686
5	Exon 7 Top Strand DNA	GST-26	Outer	GGTTTTAGTTTTTGGTTGTTGGATG (SEQ ID NO: 43)	347	50/55	3845-3869
		GST-27	Inner	TTTTTTGTTTTTAGTATATGTGGGG (SEQ ID NO: 44)	287	50/55	3874-3899
		GST-28	Inner	ATACTAAAAAACTATTTTCTAATCCTCTA (SEQ ID NO: 45)	287	50/55	4161-4132
		GST-29	Outer	CCAAACTAAAAAACCCTCCAAAAAACCACTAA (SEQ ID NO: 46)	347	50/55	4192-4164

Bases arising due to C to U conversion by bisulphite treatment are shown in bold

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TABLE 2.2 Primers for Direct Sequencing of Amplified GST-Pi Gene PCR Fragments

PCR #	Target	Primer Name	Primer Type	Primer 5' 3'	Target size (bp)	Anneal °C	Genomic Position
1	Exon 1 Top Strand DNA	GST-11	M13	TGTAAACGACGGCCAGTGGGATTGGGAAAGAGGGAA (SEQ ID NO: 47)	307	45/50	1003-1026
		GST-12	Biotin	BioACTAAAAAGCTCTAAACCCCATCCC	307	45/50	1288-1313
2	Exon 1 Bottom Strand DNA	GST-B2	M13	TGTAAACGACGGCCAGTGGGAGTTTGAGTTT (SEQ ID NO: 48)	314	50/55	999-1027
		GST-B2	Biotin	BioAAAAACCTAAAAAATAAACTTCCC (314	50/55	1285-1313

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TABLE 2.2 continued

3	Exon 2/3 Top Strand DNA	GST-14 GST-15	M13 Biotin	TGTAACGACGGCCAGT <u>TAGTATTAGGTTA</u> (SEQ ID NO: 49) BioAACTCTAACCCCTAATCTACCAACAACATA	603 603	45/50 45/50	1317-1337 1920-1892
4	Exon 4/5 Top Strand DNA	GST-31 GST-32	M13 Biotin	TGTAACGACGGCCAGT <u>TTTTGAGTATTGTTGTC</u> (SEQ ID NO: 50) BioTTAATATAAATAAAAAAATATATTTTACAA	265 265	55/60 55/60	2381-2410 2617-2646
5	Exon 7 Top Strand DNA	GST-27 GST-28	M13 Biotin	TGTAACGACGGCCAGT <u>TTTTTAGTATATGTGG</u> (SEQ ID NO: 51) BioATACTAAAAAACTATTTTCTAATCCTCTA	287 287	50/55 50/55	3874-4132 4161-4164

Extensions on "M13" primers for annealing of sequencing primer is underlined.

Primer	Forward or Reverse	Primer Sequence (5'-3')	Co-ordinates	CpG sites
CGPS-1	F	CGCGAGGTTTTCGTTGGAGTTTCGTCGTC (SEQ ID NO: 1)	1210-1238	-3 to +3
CGPS-2	F	CGTTATTAGTAGTACGCGCGGTTC (SEQ ID NO: 2)	1247-1271	+4 to +8
CGPS-3	R	TCCCATCCCTCCCGAAACGCTCCG (SEQ ID NO: 8)	1428-1452	+21 to +23
CGPS-4	R	GAAACGCTCCGAACCCCTAAACCGCTAACG (SEQ ID NO: 9)	1406-1438	+19 to +23
CGPS-5	F	YGGTTTAGGGAATTTTTTCGC (SEQ ID NO: 3)	894-917	-39 to -37
CGPS-6	F	YGGYGYGTAGTTGYGTATATTC (SEQ ID NO: 4)	925-952	-36 to -31
CGPS-11	F	GGGAATTTTTTCGCGATGTTYGGCGC (SEQ ID NO: 5)	902-930	-38 to -34
CGPS-7	R	CRCCCTAAAATCCCCRAAATCRCCGCG (SEQ ID NO: 10)	1038-1064	-23 to -27
CGPS-8	R	ACCCCRACRACCRCTACACCCCRAAACGTCG (SEQ ID NO: 11)	1077-1106	-16 to -21
CGPS-9	R	CTCTTCTAAAAAATCCCRRAACTCCCGCG (SEQ ID NO: 12)	1113-1143	-12 to -15
CGPS-12	R	AAACRCCCTAAAATCCCGGAAATCGCGC (SEQ ID NO: 13)	1040-1068	-23 to -26
CGPS-13	R	AACTCCRCGACCCGAACCCCGACGACCG (SEQ ID NO: 14)	1094-1123	-14 to -18
CGPS-21	F	TTTTTAGGGGTTYGGAGCGTTTC (SEQ ID NO: 6)	1415-1438	+21 to +23
CGPS-22	F	GGTAGGTTGYGTTTATCGC (SEQ ID NO: 7)	1473-1491	+26 to +28
CGPS-23	R	AAAAATTCRAATCTCTCCGAATAAACG (SEQ ID NO: 15)	1640-1666	+36 to +34
CGPS-24	R	AAAAACCRAAAATAAAACCCACGACG (SEQ ID NO: 16)	1676-1703	+39 to +37

TABLE 4

	Assay negative	Assay positive
Normal subjects	10	0
Benign hyperplasia	17	1
Cancer (total)	7	17
Stage A	1	3
Stage B	3	3
Stage C	0	2
Stage D	0	7
Stage not defined	3	2

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5 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Claims:

1. A diagnostic or prognostic assay for a disease or condition in a subject,
5 said disease or condition characterised by abnormal methylation of cytosine
at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its
regulatory flanking sequences, wherein said assay comprises the steps of;

(i) isolating DNA from said subject,

(ii) exposing said isolated DNA to reactants and conditions for the
10 amplification of a target region of the GST-Pi gene and/or its regulatory
flanking sequences which includes a site or sites at which abnormal cytosine
methylation characteristic of the disease or condition occurs, the
amplification being selective in that it only amplifies the target region if the
said site or sites at which abnormal cytosine methylation occurs is/are
15 methylated, and

(iii) determining the presence of amplified DNA,
wherein the amplifying step (ii) is used to amplify a target region within the
region of the GST-Pi gene and/or its regulatory flanking sequences defined by
(and inclusive of) CpG sites -43 to +55.

2. An assay according to claim 1, wherein prior to the amplifying step,
the isolated DNA is treated such that unmethylated cytosines are converted
to uracil or another nucleotide capable of forming a base pair with adenine
while methylated cytosines are unchanged or are converted to a nucleotide
25 capable of forming a base pair with guanine.

3. As assay according to any one of the preceding claims, wherein the
amplifying step involves polymerase chain reaction (PCR) amplification.

4. An assay according to claim 3, wherein said PCR amplification utilises
30 a reverse primer including guanine at at least one site whereby, upon the

reverse primer annealing to the treated DNA, said guanine will either form a base pair with a methylated cytosine (or another nucleotide to which the methylated cytosine has been converted through said treatment) if present, or will form a mismatch with uracil (or another nucleotide to which unmethylated cytosine has been converted through said treatment).

5. An assay according to claim 4, wherein said PCR amplification utilises a forward primer including cytosine at at least one site(s) corresponding to cytosine nucleotides that are abnormally methylated in the DNA of a subject with the disease or condition being assayed.

6. An assay according to claim 5, wherein the primers are of 12 to 30 nucleotides in length.

7. An assay according to claim 6, wherein the primers are selected so as to anneal to a sequence within the target region that includes two to four cytosine nucleotides that are abnormally methylated in the isolated DNA of a subject with the disease or condition being assayed.

8. An assay according to claim 2, wherein the treatment of the isolated DNA involves reacting the isolated DNA with bisulphite.

9. As assay according to claim 8, wherein the amplifying step involves polymerase chain reaction (PCR) amplification.

10. An assay according to claim 9, wherein said PCR amplification utilises a reverse primer including guanine at at least one site whereby, upon the reverse primer annealing to the treated DNA, said guanine will either form a base pair with a methylated cytosine if present, or will form a mismatch with uracil.

11. An assay according to claim 10, wherein said PCR amplification
utilises a forward primer including cytosine at at least one site(s)
corresponding to cytosine nucleotides that are abnormally methylated in the
isolated DNA of a subject with the disease or condition being assayed.

12. An assay according to claim 11, wherein the primers are of 12 to 30
nucleotides in length.

13. An assay according to claim 12, wherein the primers are selected so as
to anneal to a sequence within the target region that includes two to four
cytosine nucleotides that are abnormally methylated in the DNA of a subject
with the disease or condition being assayed.

14. An assay according to any one of the preceding claims, wherein said
DNA is isolated from cells from tissue, blood (including serum and plasma),
semen, urine, lymph or bone marrow.

15. An assay according to any one of the preceding claims, wherein the
disease or condition to be assayed is selected from cancers.

16. An assay according to claim 15, wherein the disease or condition to be
assayed is selected from prostate cancer, breast cancer, cervical cancer and
liver cancer.

17. An assay according to claim 16, wherein the disease or condition to be
assayed is prostate cancer.

18. An assay according to claim 17, wherein the amplifying step is used to
amplify a target region within the region of the GST-Pi gene and its

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regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +53.

19. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +10.

20. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -14.

21. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -8.

22. An assay according to any one of the preceding claims wherein the target region excludes any or all of the CpG sites -36, -32, -23, -20, -19 and -14.

23. An assay according to any one of claims 5 to 21, wherein if either or both of the reverse or forward primers anneal to a sequence within the target region that includes any or all of CpG sites -36, -32, -23, -20, -19 and -14, then said PCR amplification further utilises equivalent reverse and/or forward primers including a redundant nucleotide(s) at the position(s) within their sequence(s) corresponding to cytosine or methylated cytosine of CpG sites -36, -32, -23, -20, -19 and -14.

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24. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites +9 to +53.

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25. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites +1 to +53.

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26. An assay according to claim 17, wherein the amplifying step involves PCR amplification using primer pairs consisting of a forward and reverse primer selected from each of the following groups:

Forward Primers

15	CGCGAGGTTTTCGTTGGAGTTTCGTCGTC	(SEQ ID NO: 1)
	CGTTATTAGTGAGTACGCGCGGTTC	(SEQ ID NO: 2)
	YGGTTTTAGGGAATTTTTTTTCGC	(SEQ ID NO: 3)
	YGGYGYGTTAGTTYGTTGYGTATATTC	(SEQ ID NO: 4)
	GGGAATTTTTTTTCGCGATGTTYGGCGC	(SEQ ID NO: 5)
20	TTTTTAGGGGGTTYGGAGCGTTTC	(SEQ ID NO: 6)
	GGTAGGTTGYGTTTATCGC	(SEQ ID NO: 7)

Reverse Primers

	TCCCATCCCTCCCCGAAACGCTCCG	(SEQ ID NO: 8)
	GAAACGCTCCGAACCCCCTAAAAACCGCTAACG	(SEQ ID NO: 9)
25	CRCCCTAAAATCCCCRAAATCRCCGCG	(SEQ ID NO: 10)
	ACCCCRACRACRCTACACCCCRACGTCG	(SEQ ID NO: 11)
	CTCTTCTAAAAAATCCRCRAACTCCCGCCG	(SEQ ID NO: 12)
	AAAACRCCCTAAAATCCCCGAAATCGCCG	(SEQ ID NO: 13)
	AACTCCCRCCGACCCCAACCCGACGACCG	(SEQ ID NO: 14)
30	AAAAATTCRAATCTCTCCGAATAAACG	(SEQ ID NO: 15)

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AAAAACCRAAATAAAAACACACGACG (SEQ ID NO: 16),

wherein Y is C, T or a mixture thereof, and R is A, G or a mixture thereof.

27. An assay according to claim 17, wherein the amplifying step involves
5 PCR amplification using primer pairs consisting of a forward and reverse
primer selected from each of the following groups:

Forward Primers

CGCGAGGTTTTCGTTGGAGTTTCGTCGTC (SEQ ID NO: 1)

CGTTATTAGTGAGTACGCGCGGTTC (SEQ ID NO: 2)

Reverse Primers

TCCCATCCCTCCCCGAAACGCTCCG (SEQ ID NO: 8)

GAAACGCTCCGAACCCCTAAAAACCGCTAACG (SEQ ID NO: 9).

28. An assay according to claim 17, wherein the amplifying step involves
15 PCR amplification using primer pairs consisting of a forward and reverse
primer selected from each of the following groups:

Forward Primers

YGGTTTTAGGGAATTTTTTTTCGC (SEQ ID NO: 3)

YGGYGYGTTAGTTYGTTGYGTATATTC (SEQ ID NO: 4)

GGGAATTTTTTTTCGCGATGTTTYGGCGC (SEQ ID NO: 5)

Reverse Primers

CRCCCTAAAATCCCCRAAATCRCCGCG (SEQ ID NO: 10)

ACCCCRACRACCRCTACACCCRAACGTCG (SEQ ID NO: 11)

CTCTTCTAAAAAATCCCRCAACTCCCGCCG (SEQ ID NO: 12)

AAAACRCCCTAAAATCCCCGAAATCGCCG (SEQ ID NO: 13)

AACTCCCRCCGACCCCAACCCCGACGACCG (SEQ ID NO: 14),

wherein Y is C, T or a mixture thereof and R is A, G or a mixture thereof.

29. An assay according to claim 17, wherein the amplifying step involves
30 PCR amplification using primer pairs consisting of a forward and reverse

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primer selected from each of the following groups:

Forward Primers

TTTTTAGGGGGTTYGGAGCGTTTC (SEQ ID NO: 6)

GGTAGGTTGYGTTTATCGC (SEQ ID NO: 7)

Reverse Primers

AAAAATTCRAATCTCTCCGAATAAACG (SEQ ID NO: 15)

AAAAACCRAAATAAAAACCACACGACG (SEQ ID NO: 16),

wherein Y is C, T or a mixture thereof, and R is A, G or a mixture thereof.

30. An assay according to claim 16, wherein the disease or condition to be assayed is liver cancer.

31. An assay according to claim 30, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -14.

32. An assay according to claim 31, wherein the target region excludes any or all of the CpG sites -36, -32, -23, -20, -19, and -14.

33. An assay according to claim 30, wherein if either or both of the reverse or forward primers anneal to a sequence within the target region that includes any or all of CpG sites -36, -32, -23, -20, -19 and -14, then said PCR amplification further utilises equivalent reverse and/or forward primers including a redundant nucleotide(s) at the position(s) within their sequence(s) corresponding to cytosine or methylated cytosine of CpG sites -36, -32, -23, -20, -19 and -14.

34. An assay according to claim 30, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its

regulatory flanking sequences defined by (and inclusive of) CpG sites +9 to +53.

35. A diagnostic or prognostic assay for a disease or condition in a subject said disease or condition characterised by abnormal methylation of cytosine at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its regulatory flanking sequences, wherein said assay comprises the steps of;

- (i) isolating DNA from said subject, and
- (ii) determining the presence of abnormal methylation of cytosine at a site or sites within the region of the GST-Pi gene and/or its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +55.

36. An assay according to claim 35, wherein the region of the GST-Pi gene and its regulatory flanking sequences within which the presence of methylated cytosine(s) at a site or sites is determined is selected from the regions defined by (and inclusive of) CpG sites -43 to +53, -43 to +10, -43 to -14, +9 to +53 and +1 to +53.

37. An assay according to claim 35 or 36, wherein the said region of the GST-Pi gene and its regulatory flanking sequences excludes any or all of the CpG sites -36, -32, -23, -20, -19 and -14.

38. An assay according to claim 36, wherein the region of the GST-Pi gene and its regulatory flanking sequences within which the presence of methylated cytosine(s) at a site or sites is determined is the region defined by (and inclusive of) CpG sites +9 to +53.

39. An assay according to claim 36, wherein the region of the GST-Pi gene and its regulatory flanking sequences within which the presence of

methyated cytosine(s) at a site or sites is determined is the region defined by (and inclusive of) CpG sites +1 to +53.

40. An assay according to any one of claims 35 to 39, wherein prior to the determination step, the isolated DNA is treated such that unmethylated cytosines are converted to uracil or another nucleotide capable of forming a base pair with adenine while methylated cytosines are unchanged or are converted to a nucleotide capable of forming a base pair with guanine.

41. An assay according to claim 40, wherein the treatment of the isolated DNA involves reacting the isolated DNA with bisulphite.

42. An assay according to any one of claims 35 to 41, wherein the determination step involves selective hybridisation of oligonucleotide/polynucleotide/peptide-nucleic acid (PNA) probe(s).

43. An assay according to claim 42, wherein if the probe(s) hybridise to a sequence within the target region that includes any or all of CpG sites -36, -32, -23, -20, -19 and -14, then said selective hybridisations further utilises equivalent probe(s) including a redundant nucleotide(s) at the position(s) within their sequence(s) corresponding to cytosine or methylated cytosine of CpG sites -36, -32, -23, -20, -19 and -14.

44. An assay according to any one of claims 35 to 43, wherein said DNA is isolated from cells from tissue, blood (including serum and plasma), semen, urine, lymph or bone marrow.

45. An assay according to any one of claims 35 to 43, wherein the disease or condition to be assayed is selected from cancers.

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46. An assay according to claim 45, wherein the disease or condition to be assayed is selected from prostate cancer, breast cancer, cervical cancer and liver cancer.

5 47. An assay according to claim 46, wherein the disease or condition to be assayed is prostate cancer.

48. An assay according to claim 46, wherein the disease or condition to be assayed is liver cancer.

10 49. A primer or probe comprising a nucleotide sequence selected from the group consisting of:

	CGCGAGGTTTTCGTTGGAGTTTCGTCGTC	(SEQ ID NO: 1)
	CGTTATTAGTGAGTACGCGCGGTTTC	(SEQ ID NO: 2)
15	YGGTTTTAGGGAATTTTTTTTCGC	(SEQ ID NO: 3)
	YGGYGYGTTAGTTYGTTGYGTATATTTTC	(SEQ ID NO: 4)
	GGAATTTTTTTTCGCGATGTTTYGGCGC	(SEQ ID NO: 5)
	TTTTTAGGGGGTTYGGAGCGTTTC	(SEQ ID NO: 6)
	GGTAGGTTGYGTTTATCGC	(SEQ ID NO: 7)
20	AAAAATTCTAATCTCTCCGAATAAACG	(SEQ ID NO: 8)
	AAAAACCRAAATAAAAACCACACGACG	(SEQ ID NO: 9)
	TCCCATCCCTCCCCGAAACGCTCCG	(SEQ ID NO: 10)
	GAAACGCTCCGAACCCCTAAAAACCGCTAACG	(SEQ ID NO: 11)
	CRCCCTAAAATCCCCRAAATCRCCGCG	(SEQ ID NO: 12)
25	ACCCCRACRACCRCTACACCCRAACGTCCG	(SEQ ID NO: 13)
	CTCTTCTAAAAAATCCCRCAACTCCCGCCG	(SEQ ID NO: 14)
	AAAACRCCCTAAAATCCCCGAAATCGCCG	(SEQ ID NO: 15)
	AACTCCCRCCGACCCCAACCCCGACGACCG,	(SEQ ID NO: 16),

wherein Y is a mixture of C and T, and R is a mixture of A and G.

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50. A probe comprising a nucleotide sequence selected from the group consisting of:

AAACCTAAAAAATAAACAAACAA (SEQ ID NO: 17)

GGGCCTAGGGAGTAAACAGACAG (SEQ ID NO: 18)

5 CCTTTCCCTCTTTCCCAARRTCCCA (SEQ ID NO: 19)

TTTGGTATTTTTTTTCGGGTTTTAG (SEQ ID NO: 20)

CTTGGCATCCTCCCCCGGGCTCCAG (SEQ ID NO: 21)

GGYAGGGAAGGGAGGYAGGGGYTGGG (SEQ ID NO: 22).

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FIGURE 1

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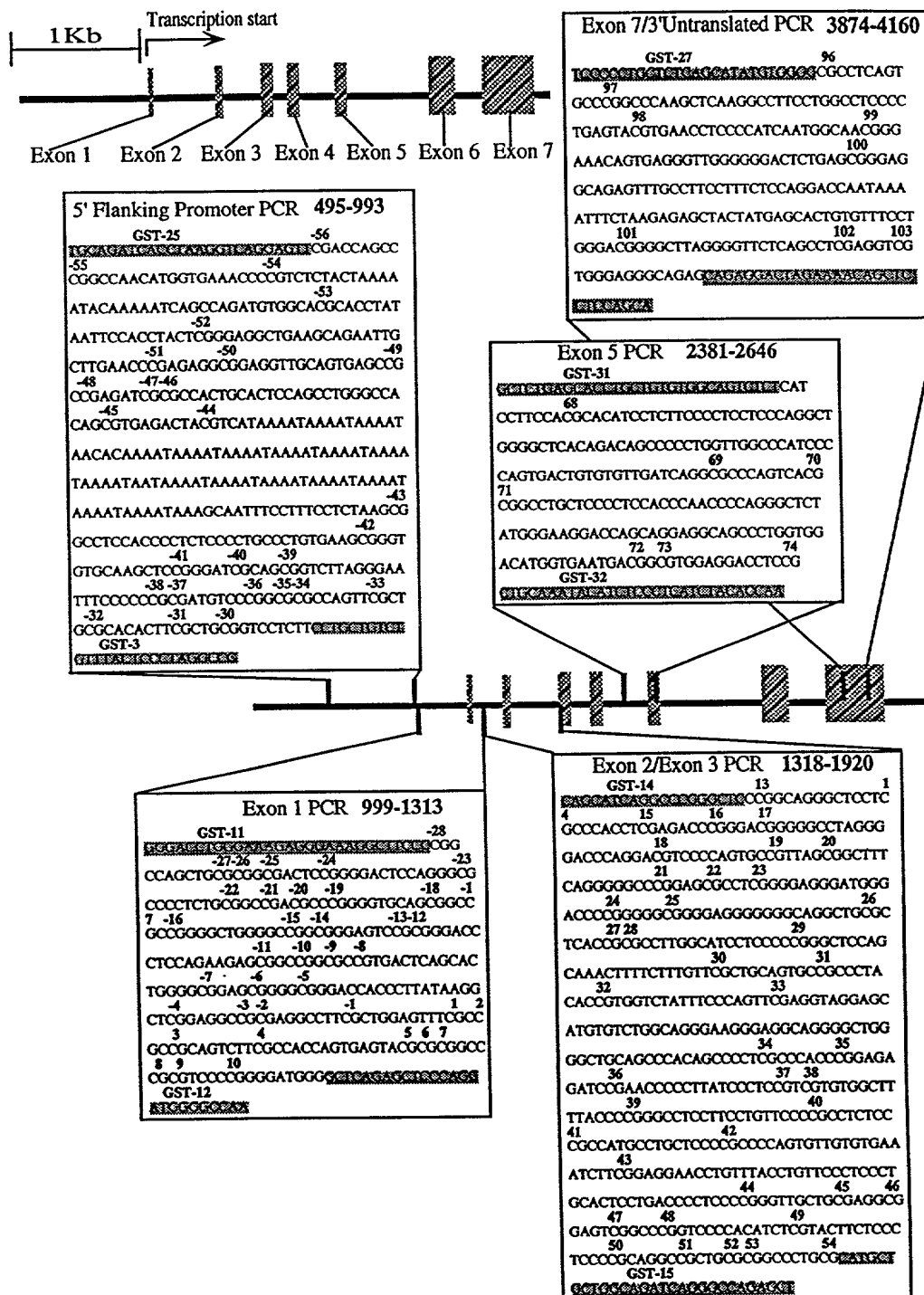


Figure 2 Upstream Region of Differential Methylation in Prostate Cancer

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Figure 2 (Continued)

```

-15 -14 -13-12 -11 -10 -9 -8 -7 -6 -5
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GTCGCGGGA GTTCGCGGA TTTTITAGAA GAGCGGTCGG GTCTGTGATT TAGTATTGGG GCGGAGCGGG GCGGGATTAT B-M

<GCCGCCCT CAARCRCCCT AAAAAATCTT CTC CGPS-9
CAGCRCCCT CAA CGPS-13

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CGPS-1 C GCGAGGTTTT CGTTGGAGTT TCGTCGTC> CGPS-2 CGTT ATTAGTGAGT ACGCGCGGTT

8 9 10
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CGCGTTTTTCG GGGATGGGT TTAGAGTTTT TAGTATGGG TTAA B-M

C>

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09/673448

PCT/AU99/00306
Received 30 June 1999

4/17

Figure 3A Methylation Status of Individual Sites in the GST-Pi Gene

site	LN	Du	PC3	PC3 M	PC3 MM	2AN	BN CN	2AC	BC	CC	DC	XC	WC	Pr
-28	+++	.	+	++	++	.	.	.	++	+	++	+++	++	.
-27	+++	.	+	++	++	.	.	.	+	+	++	+++	+	.
-26	+++	.	+	++	++	.	.	.	+	+	++	+++	++	.
-25	+++	.	+	++	++	.	.	.	+	+	++	+++	++	.
-24	+++	.	+	++	++	.	.	.	+	+	++	+++	+++	.
-23	+++	.	+	+	+	.	.	.	++	+	++	.	+++	.
-22	+++	.	++	+	+	.	.	.	++	+	++	.	+++	.
-21	+++	.	+	++	++	++	+++	++	.
-20	+++	++	++	++	.	+	.
-19	+++	.	++	+	++	++	+	+	.
-18	+++	.	++	+	++	.	.	.	++	++	++	+++	++	.
-17	+++	.	+	+	++	.	.	.	+	++	++	+++	+++	.
-16	+++	.	++	+	++	.	.	.	++	++	++	+++	+++	.
-15	+++	.	+	+	++	.	.	.	++	++	++	+++	+++	.
-14	+++	.	.	+	+	.	.	.	+	++	++	.	+	.
-13	+++	.	+	+	++	.	.	.	B	B	B	++	+	.
-12	+++	.	+	++	++	.	.	.	B	B	B	+++	+	.
-11	+++	.	++	++	++	.	.	.	+	++	++	+++	+++	.
-10	+++	.	++	++	++	.	.	.	++	++	++	+++	+++	.
-9	+++	.	++	++	++	.	.	.	++	++	++	+++	+++	.
-8	+++	.	++	++	++	.	.	.	++	++	++	+++	+++	.
-7	+++	.	++	++	++	.	.	.	++	++	++	+++	+++	.
-6	+++	.	++	++	++	.	.	.	++	++	++	+++	+++	.
-5	+++	.	++	++	++	.	.	.	++	++	++	+++	+++	.
-4	+++	.	++	++	++	.	.	.	++	++	++	+++	+++	.
-3	+++	.	++	++	++	.	.	.	++	++	++	+++	+++	.
-2	+++	.	++	++	B	.	.	.	++	++	++	+++	+++	.
-1	+++	.	++	+	B	.	.	.	++	++	++	+++	+++	.

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5/17

Figure 3A (cont'd)

1	+++	-	++	B	B	-	-	-	-	++	++	+++	+++	+++	-
2	+++	-	++	B	B	-	-	-	-	++	++	+++	+++	+++	-
3	+++	-	++	B	B	-	-	-	-	++	++	+++	+++	+++	-
4	+++	-	++	B	B	-	-	-	-	++	++	+++	+++	+++	-
5	+++	-	+++	B	B	-	-	-	-	B	++	+++	+++	+++	-
6	+++	-	+++	B	B	-	-	-	-	B	+++	+++	+++	+++	-
7	+++	-	+++	B	B	-	-	-	-	B	+++	+++	+++	+++	-
8	+++	-	++	B	B	-	-	-	-	B	++	+++	+++	+++	-
9	+++	-	++	B	B	-	-	-	-	B	++	+++	+++	+++	-
10	+++	-	++	B	B	-	-	-	-	B	++	+++	+++	+++	-
13	+++	-	+	+++	+++	+++	-	-	-	+++	+++	+++	+++	+++	-
14	+++	-	+	+++	+++	+++	-	-	-	+++	+++	+++	+++	+++	-
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25	+++	-	B	+	+	+	-	-	-	+++	+++	+++	+++	+++	-
26	+++	-	B	+	+	+	-	-	-	+++	+++	+++	+++	+++	-
27	+++	-	B	+	+	+	-	-	-	+++	+++	+++	+++	+++	-
28	+++	-	B	+	+	+	-	-	-	+++	+++	+++	+++	+++	-
29	+++	-	B	+	+	+	-	-	-	+++	+++	+++	+++	+++	-
30	+++	-	B	+	+	+	-	-	-	+++	+++	+++	+++	+++	-
31	+++	-	B	+	+	+	-	-	-	+++	+++	+++	+++	+++	-
32	+++	-	B	+	+	+	-	-	-	+++	+++	+++	+++	+++	-
33	+++	-	B	+	+	+	-	-	-	+++	+++	+++	+++	+++	-

Figure 3B Methylation Status of Individual Sites in the GST-Pi Gene

[illegible]

9/17

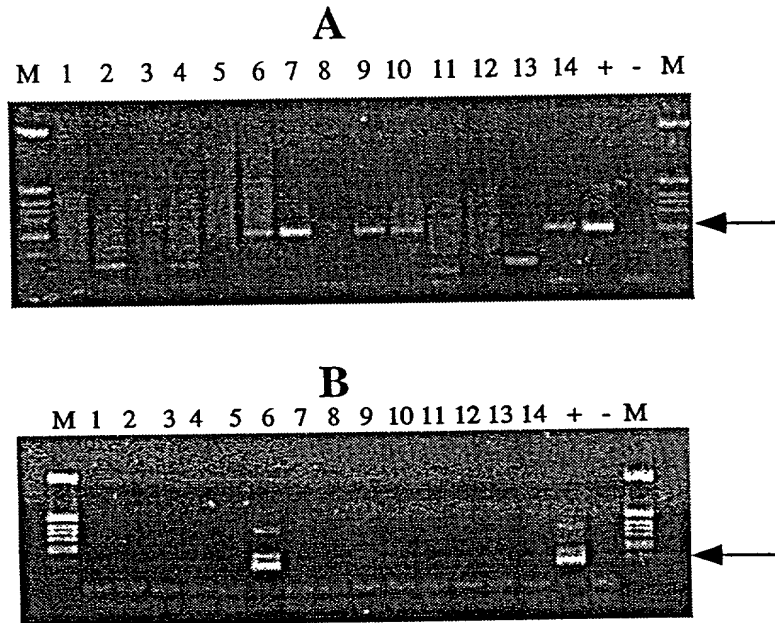
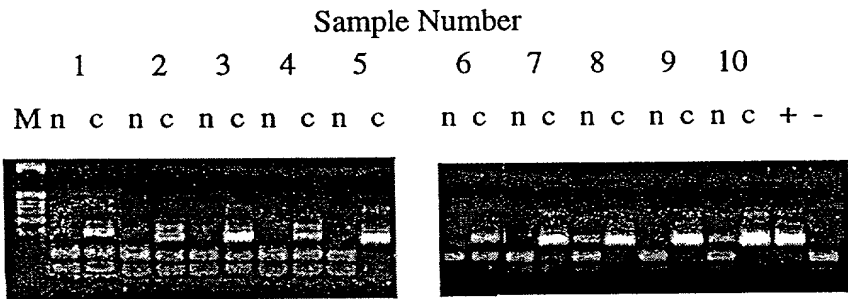


FIGURE 4A

10/17

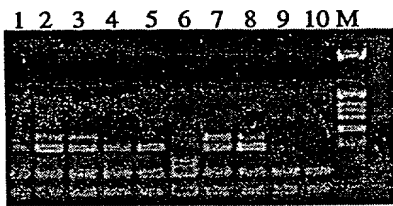


Sample	Tissue	Gleason	% Methylation Non CG rich PCR
1	Normal	N/A	-
	Cancer	3+3	++++
2	Normal	N/A	-
	Cancer	3+5	++
3	Normal	N/A	-
	Cancer	3+3	++
4	Normal	N/A	-
	Cancer	3+5	-
5	Normal	N/A	-
	Cancer	2+2	++
6	Normal	N/A	-
	Cancer	3+3	-
7	Normal	N/A	-
	Cancer	2+3	++
8	Normal	N/A	-
	Cancer	3+3	++
9	Normal	N/A	-
	Cancer	2+3	++++
10	Normal	N/A	-
	Cancer	?	++

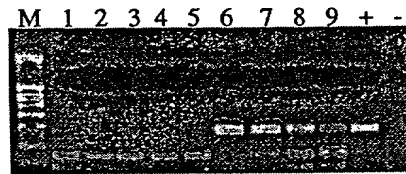
FIGURE 4B

11/17

A



B



C

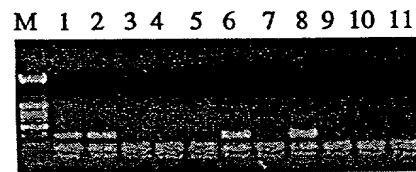
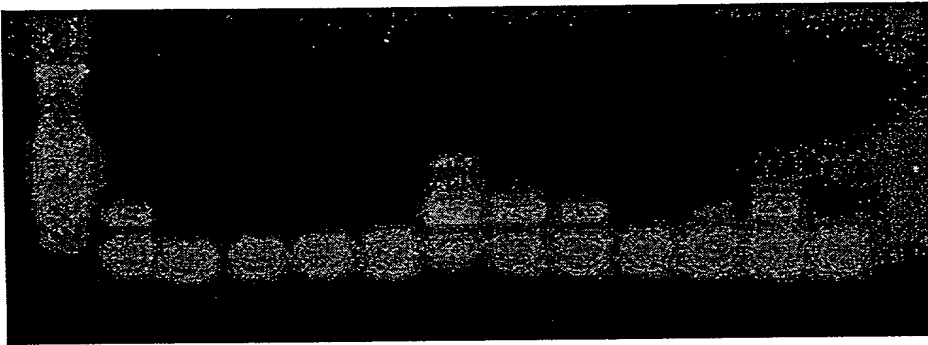


FIGURE 4C

12/17

Figure 5

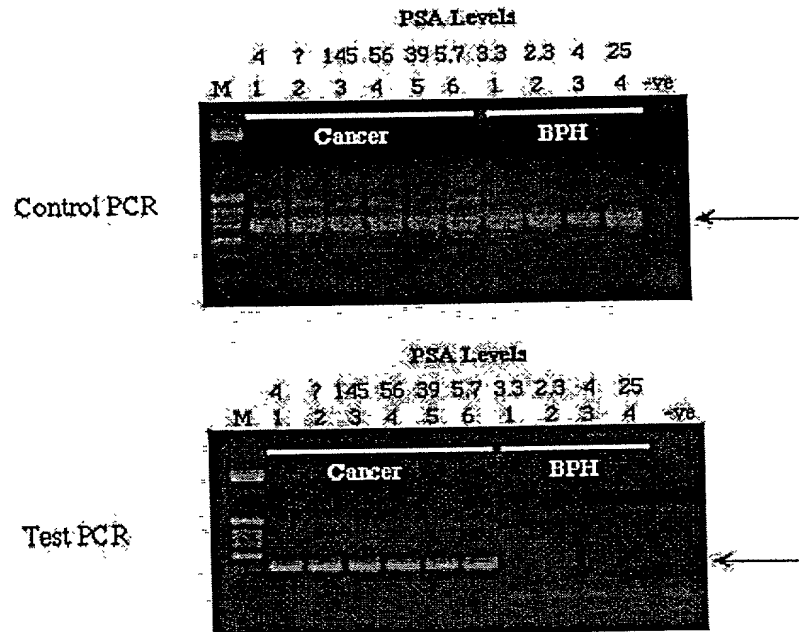
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00673448-142700

13/17

Figure 6



14/17

Figure 7A

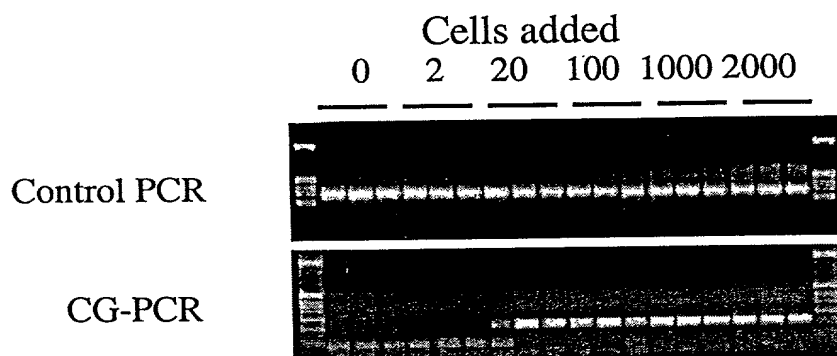
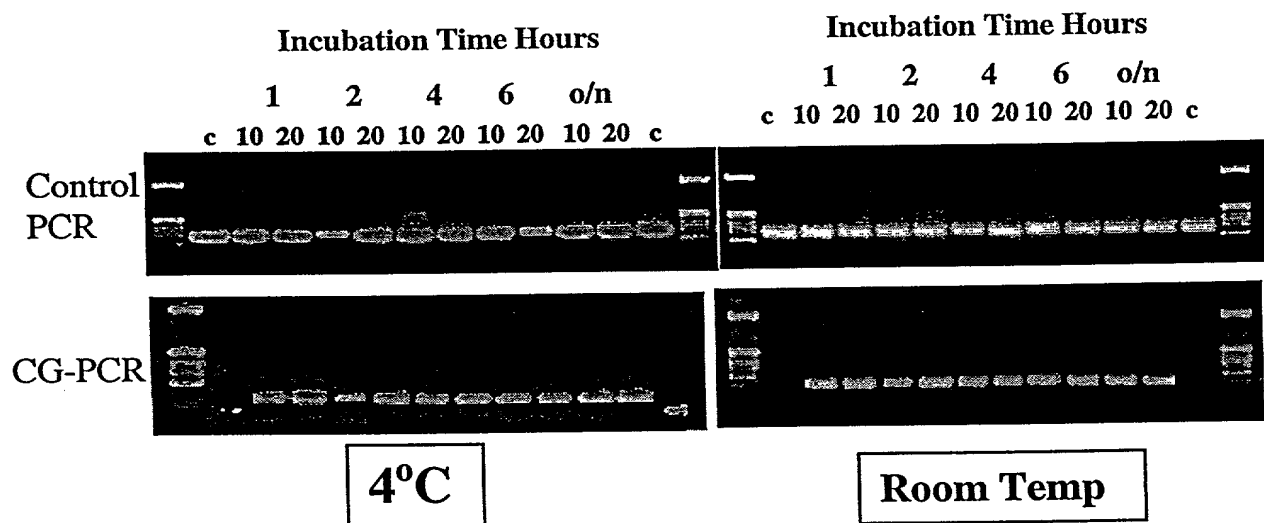
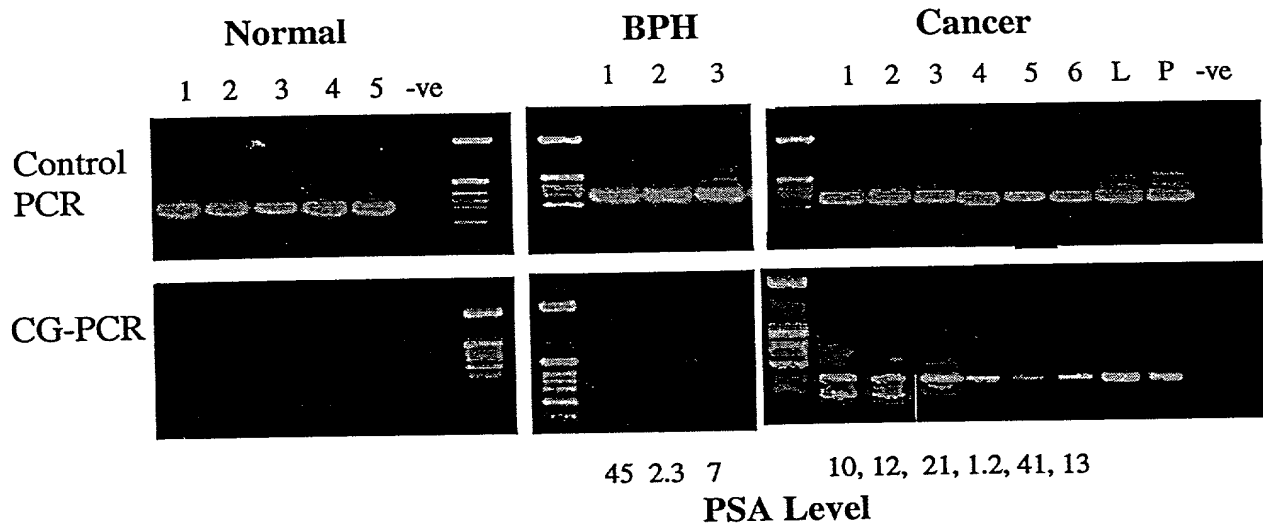


Figure 7B



15/17

Figure 8

16/17

Figure 9

Liver Cancer Tissue DNA extracts

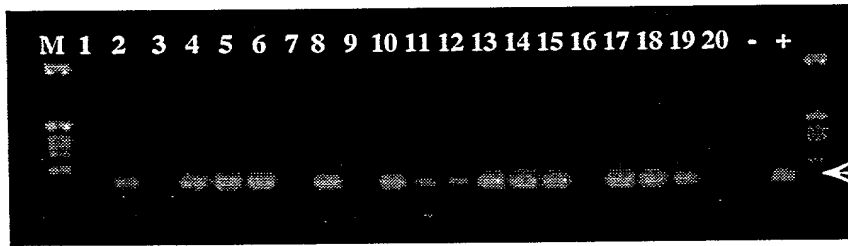
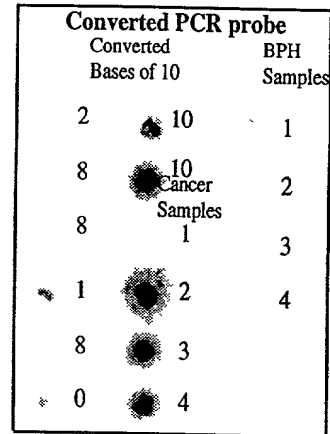
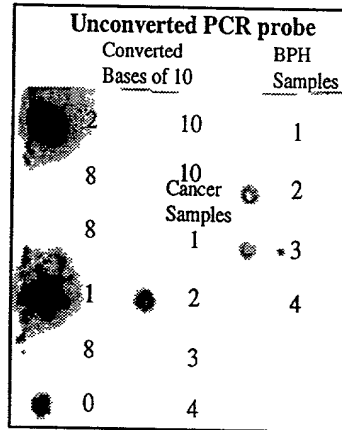
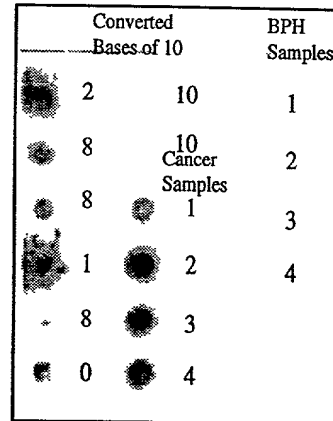


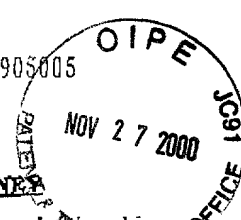
Figure 10

Test Oligo's



Control Oligo



**DECLARATION AND POWER OF ATTORNEY**

As the below named inventors, we hereby declare that our residence, post office address and citizenships are as stated below next to our names; that we verily believe we are the original and joint inventors of the subject matter claimed and for which a patent is sought in the application entitled:

ASSAY FOR METHYLATION IN THE GST-PI GENE

which application is:

☐ the attached application

(for original application)

☒ Application No. 09/673,448

filed October 16, 2000, and amended on

October 16, 2000

(for declaration not accompanying application)

that we have reviewed and understand the contents of the specification of the above-identified application, including the claims, as amended by any amendment referred to above; that we acknowledge our duty to disclose information of which we are aware which is material to the patentability of this application under 37 C.F.R. § 1.56, that we hereby claim priority benefits under Title 35, United States Code § 119, § 172 or § 365 of any provisional application or foreign application(s) for patent or inventor's certificate listed below and have also identified on said list any foreign application for patent or inventor's certificate on this invention having a filing date before that of any foreign application on which priority is claimed:

Application Number	Country	Filing Date	Priority Claimed
PCT/AU99/00306 PP 3129	PCT Australia	April 23, 1999 April 23, 1998	Yes Yes

We hereby claim the benefit of Title 35, United States Code § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in a listed prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, We acknowledge our duty to disclose any information material to the patentability of this application under 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date	Status
-----------------	-------------	--------

We hereby appoint John H. Mion, Reg. No. 18,879; Thomas J. Macpeak, Reg. No. 19,292; Robert J. Seas, Jr., Reg. No. 21,092; Darryl Moeck, Reg. No. 23,063; Robert V. Sloan, Reg. No. 22,775; Peter D. Olcay, Reg. No. 24,513; I. Frank Osha, Reg. No. 24,625; Waddell A. Biggart, Reg. No. 24,861; Louis Gubinsky, Reg. No. 24,835; Neil B. Siegel, Reg. No. 25,200; David I. Cushing, Reg. No. 28,703; John R. Inge, Reg. No. 26,916; Joseph J. Ruch, Jr., Reg. No. 26,577; Sheldon I. Landsman, Reg. No. 25,430; Richard C. Turner, Reg. No. 29,710; Howard L. Bernstein, Reg. No. 25,665; Alan J. Kasper, Reg. No. 25,426; Kenneth J. Burchfiel, Reg. No. 31,333; Gordon Kit, Reg. No. 30,764; Susan J. Mack, Reg. No. 30,951; Frank L. Bernstein, Reg. No. 31,484; Mark Boland, Reg. No. 32,127; William H. Mandir, Reg. No. 32,156; Scott M. Daniels, Reg. No. 32,562; Brian W. Hannon, Reg. No. 32,778; Abraham J. Rosner, Reg. No. 33,276; Bruce E. Kramer, Reg. No. 33,725; Paul B. Nells, Reg. No. 33,102; Brent S. Sylvester, Reg. No. 32,765; Robert M. Masters, Reg. No. 35,603 and George F. Lehnigk, Reg. No. 36,359, our attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to **SUGERUE, MION, ZINN, MACPEAK & SEAS, PLLC, 2100 Pennsylvania Avenue, N.W., Washington, D.C. 20037-3213.**

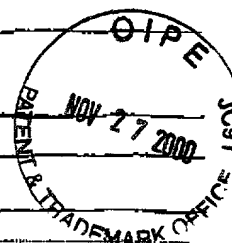
We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date 24/11/00 First Inventor Susan J. CLARK
Residence New South Wales AUSTRALIA Signature Sue Clark
City State/Country
Post Office Address: 41 Bellevue Street, Chatswood AUX
New South Wales, 2067, AUSTRALIA
Citizenship Australian

Date 24/11/00 2-00 Second Inventor Douglas S. MILLAR
First Name Middle Initial Last Name
Residence New South Wales AUSTRALIA Signature [Signature]
City State/Country

Post Office Address: 56 Trafalgar Parade, Concord AUX
New South Wales, 2137, AUSTRALIA

Citizenship Great Britain



Date November 24, 2000 3-00 Third Inventor Peter L. MOLLOY
First Name Middle Initial Last Name
Residence New South Wales AUSTRALIA Signature P.L. Molloy
City State/Country

Post Office Address: 41 Bellevue Street, Chatswood AUX
New South Wales, 2067, AUSTRALIA

Citizenship Australian

Date _____ Fourth Inventor _____
First Name Middle Initial Last Name
Residence _____ Signature _____
City State/Country

Post Office Address: _____

Citizenship _____

Date _____ Fifth Inventor _____
First Name Middle Initial Last Name
Residence _____ Signature _____
City State/Country

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Citizenship _____

Date _____ Sixth Inventor _____
First Name Middle Initial Last Name
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SEQUENCE LISTING

<110> CLARK, Susan J.
MILLER, Douglas S.
MOLLOY, Peter L.

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<210> 38	
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<213> Homo sapiens	
<400> 38	
caaaaaactt taaataaacc ctctacca	29

<210> 39
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<212> DNA
<213> Homo sapiens

<400> 39
gttttgggt taggtgttt ttaggtgt ag 32

<210> 40
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<212> DNA
<213> Homo sapiens

<400> 40
gttttgagta tttgttgtgt gtagttttt 30

<210> 41
<211> 30
<212> DNA
<213> Homo sapiens

<400> 41
ttaatatataaa taaaaaaaaat atatttaca 30

<210> 42
<211> 34
<212> DNA
<213> Homo sapiens

<400> 42
taacccccaa tacccaaccc taatacaaat actc 34

<210> 43
<211> 26
<212> DNA
<213> Homo sapiens

<400> 43
ggtttttagtt tttggttggt tggatg 26

<210> 44
<211> 26
<212> DNA
<213> Homo sapiens

<400> 44
tttttttgggt ttttagtatat gtgggg 26

<210> 45

<211> 30
<212> DNA
<213> Homo sapiens

<400> 45
ataactaaaa aactattttc taatcctcta

30

<210> 46
<211> 29
<212> DNA
<213> Homo sapiens

<400> 46
ccaaactaaa aactccaaaa aaccactaa

29

<210> 47
<211> 38
<212> DNA
<213> Artificial/Unknown

<220>
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<222> ()..()
<223> M13-human GST-Pi oligonucleotide

<400> 47
tgtataaacga cggccagtgg gatttgggaa agagggaa

38

<210> 48
<211> 38
<212> DNA
<213> Artificial/Unknown

<220>
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<222> ()..()
<223> M13-human GST-Pi oligonucleotide

<400> 48
tgtataaacga cggccagttg ttgggagttt tgagtttt

38

<210> 49
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<212> DNA
<213> Artificial/Unknown

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<223> M13-human GST-Pi oligonucleotide

<400> 49
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<212> DNA
<213> Artificial/Unknown

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<212> DNA
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gaggctgaag cagaattgct tgaacccgag aggcggaggt tgcagtgagc cgccgagatc 180
gcgccactgc actccagcct gggccacagc gtgagactac gtcataaaat aaaataaaat 240
aacacaaaat aaaataaaat aaaataaaat aaaataaaat aataaaaataa aataaaaataa 300
aataaaaataa aataaaaataa agcaatttcc tttcctctaa gcggcctcca cccctctccc 360
ctgccctgtg aagcgggtgt gcaagctccg ggatcgcagc ggtcttaggg aatttcccc 420
cgcgatgtcc cggcgcgcca gttcgctgcg cacacttcgc tgcggtcctc ttcctgctgt 480

ctgtttactc cctaggccc

499

<210> 53
<211> 316
<212> DNA
<213> Homo sapiens

<400> 53
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agggcgcccc tctgcggccg acgcccgggg tgcagcggcc gccggggctg gggccggcgg 120
gagtccgcgg gaccctccag aagagcggcc ggcgccgtga ctcagcactg gggcggagcg 180
gggcgggacc acccttataa ggctcggagg ccgcgaggcc ttcgctggag ttccgcccgc 240
gcagtcttcg ccaccagtga gtacgcgcgg cccgcgtccc cggggatggg gctcagagct 300
gcagcatgg ggccaa 316

<210> 54
<211> 603
<212> DNA
<213> Homo sapiens

<400> 54
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cctaggggac ccaggacgtc cccagtgccg ttagcggctt tcagggggcc cggagcgctc 120
cggggaggga tgggaccccg ggggcgggga gggggggcag gctgcgctca ccgcgccttg 180
gcctcctccc ccgggctcca gcaaactttt ctttgttcgc tgcagtgccg ccctacaccg 240
tggtctatatt cccagttcga ggtaggagca tgtgtctggc aggggaaggga ggcaggggct 300
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tcgtgtggct tttaaccccg gcctccttcc tgttccccgc ctctcccgcc atgcctgctc 420
ccgccccag tgttgtgtga aatcttcgga ggaacctgtt tacctgttcc ctccctgcac 480
tcctgacccc tccccgggtt gctgcgaggg ggagtcggcc cggccccac atctcgact 540
tctccctccc cgcaggccgc tgcgcggccc tgcgcatgct gctggcagat cagggccaga 600
gct 603

<210> 55
<211> 266
<212> DNA
<213> Homo sapiens

<400> 55

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atcaggcgcc cagtcacgCG gcttgcctcc ctccacccaa ccccagggtt ctatgggaag	180
gaccagcagg aggcagccct ggtggacatg gtgaatgacg gcgtggagga cctccgctgc	240
aaatacatct ccctcatcta caccaa	266

<210> 56
 <211> 287
 <212> DNA
 <213> Homo sapiens

<400> 56	
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tgccctcccc tgagtacgtg aacctcccca tcaatggcaa cgggaaacag tgaggggttg	120
ggggactctg agcgggaggg agagtttgcc ttcctttctc caggaccaat aaaatttcta	180
agagagctac tatgagcact gtgtttcctg ggacggggct taggggttct cagcctcgag	240
gtcgggtggga gggcagagca gaggactaga aaacagctcc tccagca	287

<210> 57
 <211> 524
 <212> DNA
 <213> Homo sapiens

<400> 57	
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cccccgcgat gtcccggcgc gccagttcgc tgcgcacact tcgctgcggt cctcttcctg	180
ctgtctgttt actccctagg ccccgctggg gacctgggaa agagggaag gcttccccgg	240
ccagctgcgc ggcgactccg gggactccag ggcgccccctc tgcggccgac gcccggggtg	300
cagcggccgc cggggctggg gccggcgggga gtccgcggga ccctccagaa gagcggccgg	360
cgccgtgact cagcactggg gcggagcggg gcgggaccac ccttataagg ctcgagggcc	420
gcgaggcctt cgctggagtt tcgcgcgcgc agtcttcgcc accagtgagt acgcgcggcc	480
cgcgtccccg gggatggggc tcagagctcc cagcatgggg ccaa	524

<210> 58
 <211> 524
 <212> DNA
 <213> Homo sapiens

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<400> 58
ataaaataaaa ataaaataaaa ataaagtaat tttttttttt ttaagtgggt tttatttttt 60
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tttttgtgat gttttgggtgt gttagtttgt tgtgtatatatt ttgttgtgggt tttttttttg 180
ttgtttgttt attttttagg ttttgttggg gatttgggaa agagggaaaag gtttttttgg 240
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tggtgtgatt tagtattggg gtggagtggg gtgggattat ttttataagg tttggaggtt 420
gtgaggtttt tgttggagtt ttgttgttgt agtttttgtt attagtgagt atgtgtgggt 480
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<210> 59
<211> 524
<212> DNA
<213> Homo sapiens

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<400> 59
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tttttcgcgat gtttcggcgc gttagttcgt tgcgtatatatt tcgttgcggt tttttttttg 180
ttgtttgttt attttttagg tttcgttggg gatttgggaa agagggaaaag gtttttttcgg 240
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tagcggtcgt cgggggttggg gtcggcggga gttcgcggga ttttttagaa gagcggtcgg 360
cgctgtgatt tagtattggg gcggagcggg gcgggattat ttttataagg ttcggaggtc 420
gcgaggtttt cgttggagtt tcgtcgtcgt agtttttcgt attagtgagt acgcgcgggt 480
cgcgtttttc gggatggggg ttagagtttt tagtatgggg ttaa 524

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